ABSTRACT

Title of Document: REGULATION OF MACROAUTOPHAGY BY VITAMIN A/ RETINOIDS

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Retinoic acids (RAs) have diverse biologic effects and regulate several cellular functions. Here, we investigated the role of RA on autophagy by studying its effects on autophagosome (AUT) maturation, as well as on upstream regulators of autophagosome biogenesis. Our studies, based on the use of pH-sensitive fluorescent reporter markers, suggest that RA promotes AUT acidification and maturation. By using competitive inhibitors and specific agonists, we demonstrated that this effect is not mediated by the classic Retinoic Acid Receptor (RAR) and Retinoid X Receptors (RXR). RA did not affect the protein expression levels of upstream regulators of autophagy, such as Beclin-1, phospho-mTOR, and phospho-Akt1, but induced redistribution of both endogenous cation-independent mannose-6-phosphate receptor CI-MPR and transiently transfected GFP and RFP full-length CI-MPR fusion proteins from the trans-Golgi region to acidified AUT structures. Those structures were found to be amphisomes (acidified AUTs) and not autophagolysosomes. The critical role of CI-MPR in AUT maturation was further demonstrated by siRNA-mediated silencing of endogenous CI-MPR. Transient CI-MPR knockdown resulted in remarkable
accumulation of nonacidified AUTs, a process that could not be reversed with RA. These results suggest that RA induces AUT acidification and maturation by regulating CI-MPR subcellular location, a process critical in the cellular autophagic mechanism.
REGULATION OF MACROAUTOPHAGY BY VITAMIN A/RETINOIDS

By

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Dissertation submitted to the Faculty of the Graduate School of the
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2013

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Dedication

I wholeheartedly dedicate this work to my parents for their perseverance and patience, my brother and sister for their sacrifices, to my wife for her love and belief in my abilities and to my dreams and curiosities, which have made me the person I am today.
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Throughout the course of my thesis work, I have been fortunate enough to interact with a number of people who have encouraged me, believed in me, and helped me to believe that I was capable of doing and achieving much more than I ever thought I could. These people are not only brilliant scientists but also, great human beings who have really helped me over the years. The satisfaction and euphoria of successful completion of my doctoral studies would be incomplete, without the mention of the people who made it possible and whose constant guidance and encouragement served as a beacon of light. Thus, I consider it as my privilege to express through the pages of this study a word of gratitude and respect to all those personality who inspired me in the completion of this study.

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List of Abbreviations

aa amino acid

AKT1 RAC-α serine/threonine-protein kinase

ATRA all trans-retinoic acid

ATRA all trans-retinoic acid

CFP cyan fluorescent protein

CI-MPR cation-independent mannose 6-phosphate receptor

DABCO 1,4-diazabicyclo[2.2.2]octane

DMSO dimethyl sulfoxide

FACS fluorescence-activated cell sorting

HRP horseradish peroxidase

IGFII insulin-like growth factor 2

IGFIIR insulin-like growth factor 2 receptor

LAMP1 lysosomal-associated membrane protein 1

LC3 light chain 3

M6P mannose-6-phosphate

M6PR mannose-6-phosphate receptor

mGFP monomeric green fluorescent protein

mRFP monomeric red fluorescent protein

mTOR mammalian target of rapamycin
ORF open reading frame
PI3K phosphoinositide 3-kinase
RA retinoic acid
RAR retinoic acid receptor
RIPA radioimmunoprecipitation
RXR retinoid X receptor
SDS sodium dodecylsulfate
SEM standard error of the mean
siRNA small interfering RNA
TGN trans-Golgi network
LIST OF PUBLICATIONS USED IN THIS THESIS


Chapter I

General Introduction
Introduction

Eukaryotic cells are equipped with several degradation systems, the predominant system being the evolutionarily conserved process of autophagy. Basal autophagy plays critical role in cell homeostasis, as it is responsible for the degradation of excessive, malfunctioning and damaged organelles and proteins, and whole invading organisms such as bacteria and viruses (Reggiori and Klionsky, 2002; Wang and Klionsky, 2003). In addition to basal functions, autophagy provides an alternative energy source and a temporary survival mechanism under stress conditions (Rajawat and Bossis, 2008). Autophagy is readily induced in response to metabolic and endoplasmic reticulum stress, radiation and anticancer drugs, and may play an active role in initiating apoptosis and cell death (Yorimitsu et al., 2006; Ito et al., 2005; Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007; Baehrecke, 2005). Autophagy is a complex phenomenon controlled by several regulatory proteins and signaling pathways. The process relies on formation of double-membrane vesicular structures called autophagosomes (AUTs) that engulf the material destined for degradation (cargo selection). Initially formed AUTs cannot degrade the selected cargo. In subsequent and largely unknown maturation steps, AUTs become acidified, lose the inner autophagosomal membrane (amphisomes) or fuse with lysosomes (autolysosomes) (Rajawat et al., 2009). The low pH and the presence of hydrolases in acidified AUTs, amphisomes and autolysosomes allows for digestion of the cargo material. Although this cellular process was initially described over 50 years ago, we have only recently started unraveling the basic mechanisms of autophagy and understanding its role in various pathophysiological conditions.
Several human diseases are associated with decreased autophagic activity, particularly in nondividing cells of the nervous and muscle systems, where turnover of intracellular proteins may be critical. Muscular vacuolar disorders (X-linked myopathy, inclusion body myositis, and Marinesco-Sjögren syndrome) are associated with massive accumulation of imperfect AUTs or lysosomes (Nishino, 2003; Nishino et al., 2000; Kalimo et al., 1988; Nonaka, 1999). Danon disease (cardiomyopathy and mental retardation) is caused by a deficiency in LAMP-2 which is involved in endosomal and lysosomal fusion during the late maturation phase of AUTs (Nishino et al., 2000). Reduced autophagic activity and accumulation of protein aggregates is also found in neurodegenerative disorders, such as Parkinson's, Alzheimer's, Huntington's and transmissible spongiform encephalopathy diseases (Anglade et al., 1997; Cataldo et al., 1996; Kegel et al., 2000; Liberski et al., 2004). Reduced autophagy has also been suggested to increase susceptibility to cancer development and progression, however the mechanism is unclear. One could hypothesize that perturbations at any stage of AUT formation, maturation, or fusion would result in progressive accumulation of aggregated and damaged structures. Those damaged components are a likely source of oxidative-stress which in turn results in DNA damage and increased tumorigenicity. The prediction would then be that induction of autophagy may be used for cancer prevention, similarly to the rapidly evolving use of autophagy stimulators for prevention of neurodegenerative disorders (Sarkar and Rubinsztein, 2008). In addition to the classic aging disorders, autophagy has also been implicated in pathogenesis of infectious diseases. In general, autophagy functions as a protective mechanism against infections. Most foreign invaders
(bacteria, parasites, viruses) must actually bypass the autophagic line of defense before they can establish infections (Dorn et al., 2002; Tallóczy et al., 2002). Recent immunological studies have also indicated the importance of autophagy in antigen presentation and successful cellular defense. It can be speculated that the decline in autophagic activity with age may contribute to the apparent increased risk of infectious disease in the elderly.

Nutrient availability is one of the best characterized factors involved in regulation of autophagy. In general, macronutrient (proteins, carbohydrates, lipids) availability suppresses autophagy. Macronutrient deprivation and especially reduction in certain glucogenic amino acids, mainly glutamine, triggers autophagic protein degradation, while the presence of some amino acids (leucine, tyrosine and phenylalanine) is sufficient to inhibit autophagy in various cells (Schworer and Mortimore, 1979; Mordier et al., 2000; Kanazawa et al., 2004). Carbohydrates don’t directly affect autophagy; however, they may act indirectly through effects on insulin and insulin receptor. Carbohydrates are mostly broken down and become available to cells as glucose. An increase in systemic glucose concentration induces insulin secretion which causes activation of the insulin receptor and mTOR and suppression of autophagy (Ebato et al., 2008). Lipids can affect autophagy indirectly through the insulin/glucagon signaling pathway and cholesterol metabolism, or directly, as increased intracellular lipid content has been recently shown to impair autophagy (Cheng et al., 2006; Singh et al., 2009). With the exception of few reports on vitamin D3, E and K2, the effect of micronutrients on autophagy has not been extensively studied. In these reports, treatment of pancreatic stellate cells with vitamin E, HL-60
leukemia cells with vitamin K2, and MCF-7 breast cancer cells with vitamin D3 induced an increase in the number of AUTs (Høyer-Hansen et al., 2005; Ohtani et al., 2007; Rickmann et al., 2007; Yokoyama et al., 2008). The mechanism by which vitamins E and K2 increased autophagy in the previous studies has not been reported. Induction of autophagy by vitamin D3 and analogues was found to be mediated by an increase in intracellular calcium levels which in turn affect the mTOR signaling cascade (Høyer-Hansen et al., 2005).

The importance of VitA as an essential dietary component has been recognized a century ago (McCollum and Davis, 1913; Osborne and Mendel, 1919). The vitamin is essential in several physiological processes such as reproduction, embryonic development, tissue remodeling, immune function and vision (Underwood and Arthur, 1996; Mangelsdorf et al., 1994). VitA deficiency has been historically considered as a nutritionally acquired immunodeficiency disease. Even children who are only mildly deficient in VitA have a higher incidence of infectious diseases and a higher rate of mortality compared to children who consume sufficient VitA (Field et al., 2002). VitA supplementation has been found to decrease both the severity and incidence of deaths related to diarrhea and measles in developing countries, where vitamin A deficiency is common (West, 2000). The mechanism however by which VitA protects against viral and bacterial infections is still unknown. Natural and synthetic retinoids are also known to play a major role in regulating cellular growth, and differentiation, and can induce apoptosis in a wide variety of malignant cell types (Ahuja et al., 1997; Delia et al., 1993; O'Connell et al., 1996). More than 80 years ago, it was first discovered that vitamin A deficiency in rodents causes squamous
metaplasia in the trachea and other epithelial sites that could be reversed by vitamin A supplementation (Wolbach et al., 1925). Despite extensive studies, the mechanism by which retinoids suppress tumorigenesis is still largely unknown.

Retinoids are a class of natural and synthetic vitamin A analogues structurally related to All-trans Retinoic Acid (ATRA) and 9-cis-RA (the major active metabolites of Vitamin A). Retinoids exert most of their effects by binding to specific nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). ATRA binds and activates the RAR receptors, while 9-cis-RA binds and activates both RAR and RXR receptors (Teboul et al., 2008). Recent evidence though suggests that other retinoid response pathways that are independent of the nuclear receptors may exist (Mologni et al., 1999; Clifford et al., 1999; Kim et al., 1995). Interestingly, photoaffinity labeling studies has shown direct binding of ATRA to the cation-independent mannose-6-phosphate/IGFII receptor (CI-MPR) with high affinity (Kang et al., 1997), however, the binding site for ATRA is not known. CIMPR is a ubiquitously and constitutively expressed glycoprotein that plays fundamental role in endocytosis and degradation of extracellular ligands (IGF-II, uPAR), lysosomal enzyme trafficking and regulation of apoptotic and mitogenic effects (El-Shewy et al., 2009). The primary function of CIMPR is to sort and transport mannose-6-phosphate (M6P) bearing glycoproteins (such as hydrolases) from TGN to endosomes/lysosomes., however it is distinct from those for M6P and Insulin like Growth Factor –II (IGF-II) on the receptor (El-Shewy et al., 2009).

Owing to their ability to regulate aberrant cell growth, retinoids are currently being evaluated as preventive or therapeutic agents in a variety of human premalignancies
and cancer (Altucci et al., 2007; Kast, 2008; Dragnev et al., 2003). In current oncologic practice, ATRA is used to induce remission of acute promyelocytic leukemia, juvenile chronic myelogenous leukemia and mycosis fungoides. Unfortunately, the ability of retinoids to regulate cellular processes \textit{in vivo} is associated with high incidence of undesirable side effects. Consequently, aggressive use of retinoids in oncology or wider use in other diseases (psoriasis, acne) has been precluded by unacceptable side effects including lipid and bone toxicity, visual effects, and teratogenicity (Chroni et al., 2010; Moise et al., 2007). Therefore, there is a great need to develop new retinoid-related compounds capable of activating selective mechanisms but devoid of undesirable side effects.
Working Hypothesis and Research Objective

During the last two decades, considerable advances have been made in understanding the cellular signaling pathways that regulate autophagy, and manipulation of nutrient availability has been the primary experimental methodology to study the phenomenon. In this present research we aim to study the regulation of Macroautophagy process by retinoids and to determine the effects of dietary VitA on autophagy in various stages of development. Pharmacological modulation of disrupted autophagic activity has been suggested as a strategy for therapies within a wide spectrum of pathological situations including cancer, neurological diseases, premature aging and infectious diseases. Although retinoids are a multi-targeting class of compounds that can modulate several physiological and cellular processes, this research study aims to shed light on retinoid regulation of the process of Macroautophagy. Novel findings from this study can lay the foundation for the development of new and specific retinoid analogues that could enhance or reduce autophagic activity.

Research Objective

A. Evaluation of the effects of Vitamin A (All-trans Retinoic Acid, ATRA) on autophagy in vitro

A1) Generation of Hela CFP-LC3 (Microtubule associated protein Light Chain-3) Stable cell line.

A2) Generation of CFP-LC3 and mCherry-LC3 fusion proteins.
A3) To evaluate the effect of ATRA treatment on HeLa cells or Stable cell line.

A4) Evaluation of the effect of ATRA treatment on upstream regulator of autophagy.

B) To assess the involvement of receptors in physiologic outcome of ATRA treatment on HeLa cells.

B1) To assess the involvement of classic RAR nuclear receptors on biological outcome of ATRA treatment on HeLa cells or Stable cell line

B2) Generation of the GFP and mRFP cation independent mannose-6-phosphate receptor (CI-MPR) fusion protein and evaluate the effect of ATRA treatment on cellular expression or distribution of CI-MPR

B3) To assess the biological effect of retinoids on autophagy via CI-MPR
Chapter II
Review of Literature
Intracellular proteolytic systems

The degradation of intracellular proteins and other cytosolic components plays a very important role in the maintenance of intracellular homeostasis, removal of damaged and abnormal proteins, cellular differentiation and remodeling, and in cellular defense. A balance between protein synthesis and degradation is always maintained in eukaryotic and prokaryotic systems (Cuervo, 2004; Goldberg, 2003; Ciechanover, 2005; Mizushima et al., 2008). Perturbation of one of these processes can lead to the destruction of the balance and can have a detrimental effect on cell survival. Proper turnover (synthesis and degradation) of proteins is essential for normal cellular function. Cells contain different proteolytic systems, some of which are responsible for general degradation of proteins, while others are involved in proteolysis of smaller protein subsets or active only in particular cellular compartments. Quantitatively, the most important proteolytic pathways inside cells are the ubiquitin-proteasome pathway and the lysosomal pathway. With few notable exceptions, most short half-life proteins are degraded by the proteasome, whereas most of the long half-life proteins are degraded in lysosomes (Ciechanover, 2005).

There are various pathways by which lysosomes receive the extracellular and intracellular material that is to be degraded (Fig. 1) (Dice, 2000). Endocytosis can internalize extracellular proteins, as well as integral membrane proteins and sequester them in endosomes. Subsequently, these endosomes fuse with lysosomes and deliver the cargo (Luzio et al., 2007). Crinophagy is the process by which secretory proteins are delivered to lysosomes by fusion with secretory vesicles (Glaumann, 1989).
Autophagy is the bulk degradation of cytoplasm and organelles by promoting the transfer of material

**Fig.1 The lysosome-mediated cellular degradative Process:** Lysosomes are responsible for digestion of both exogenous material and endogenous proteins and cellular organelles. Exogenous proteins are targeted to the lysosome through phagocytosis, pinocytosis, and receptor-mediated endocytosis. Endogenous proteins and cellular organelles are targeted by microautophagy, macroautophagy and chaperone-mediated autophagy (CMA). The various intermediate structures and organelles (endosomes, autophagosomes, multivesicular bodies) are mostly devoid of hydrolases. CMA: chaperone-mediated autophagy. RLC: receptor ligand complex. RME: receptor-mediated endocytosis. MVB: multivesicular bodies.
from one, topologically distinct compartment, to another from the cytosol to the vacuole in yeast or to lysosomes in eukaryotic cells (Mizushima et al., 2008). Chaperone-mediated autophagy (CMA) is a more selective degradation process of autophagy in which a specific sequence in the target substrate protein is recognized by cytoplasmic molecular chaperones. Subsequently, the substrate-chaperone complex is delivered to lysosome for degradation (Dice, 2007).

Lysosomes are the major organelles responsible for proteolytic degradation and recycling. The main function of these microscopic organelles is the digestion of macromolecules derived from phagocytosis, endocytosis and autophagy (Fig. 1). Autophagy is a catabolic process that eliminates aggregates of aberrant proteins, superfluous or damaged organelles and sometimes even entire organisms such as, invading bacteria. Although, autophagosomes possess some hydrolytic activity, this activity is insufficient to complete proteolytic degradation. Thus, fusion of autophagosomes with lysosomes can be viewed as the Achilles heel in the process of autophagy.

Proteasome

The well-characterized ubiquitin-proteasomal system degrades substrate proteins tagged with a poly-ubiquitin chain. Cytosolic proteins destined for proteasomal degradation display covalently attached ubiquitin moieties or ubiquitin chains linked through the Lysine-48 (K-48) residue of ubiquitin. Ubiquitination involves three sequentially acting enzymes: E1 which is the ubiquitin activating enzyme, E2 or ubiquitin conjugating enzyme and E3, the ubiquitin-ligase, which
transfers the activated ubiquitin to the substrate. These poly ubiquitinated substrates are targeted to the major cytosolic protease, the 26S proteasome, which consists of a 20S core particle (CP) and a 19S regulatory complex (RP). The 20S CP is a proteolytic cylindrical chamber with catalytic sites for different types of proteolytic activities required for degradation of the substrate proteins. The 19S regulatory unit sits on top of the catalytic core and allows recognition of potential targets (ubiquitinated proteins). The different subunits of the 19S domain also assist in the removal of the ubiquitin from substrate proteins, as well as their subsequent unfolding and active internalization into the catalytic chamber (Ciechanover, 2006). The ubiquitin-proteasome system plays a critical regulatory role in essential cellular processes such as cell division, cell cycle, and cellular differentiation due to its ability to rapidly modulate the levels of transcription factors and signaling proteins.

**Autophagy**

Autophagy (derived from the Greek meaning “to eat oneself”) is present in all eukaryotic cells and is evolutionarily conserved from yeast to humans (Levine and Klionsky, 2004). Autophagy is a ubiquitous catabolic process that involves the bulk degradation of cytoplasmic components through a lysosomal pathway. This process is characterized by the engulfment of part of the cytoplasm inside double-membrane vesicles called autophagosomes. Autophagosomes subsequently fuse with lysosomes to form an autophagolysosome in which the cytoplasmic cargo is degraded and the degradation products are recycled for the synthesis of new molecules (Lee and Marzella, 1994). Turnover of most long-lived proteins, macromolecules, biological
membranes, and whole organelles, including mitochondria, ribosomes, endoplasmic reticulum and peroxisomes, is mediated by autophagy (Cuervo, 2004). The autophagic machinery actually mediates the majority of intracellular housekeeping tasks. Short-lived nuclear and cytoplasmic proteins are degraded by multicatalytic proteinase complexes (proteasomes) and by calcium-dependent neutral proteases (calpains). Mitochondria can autonomously turn over certain defective proteins, because they possess their own proteolytic systems (Bakala et al., 2003; Arnold and Langer, 2002)

The cellular process of autophagy was initially described over 50 years ago. Only recently though, we have started understanding its role in various pathophysiological conditions and unraveling the basic cell biology mechanism. Discovery of the ATG genes in yeast has greatly advanced our understanding of the molecular mechanisms involved in autophagosome biogenesis and the various pathways that regulate autophagic activity (Suzuki and Ohsumi, 2007). Most yeast ATG genes have orthologues, in multicellular organisms (nematodes, flies) and mammals, which suggests that the basic machinery for autophagy has been evolutionarily conserved in eukaryotic organisms. These genes, also called APG/AUT/CVT, were initially identified from studies that investigated defects in the formation of autophagic vacuoles and protein degradation in Saccharomyces cerevisiae (Takeshige et al., 1992; Thumm et al., 1994). In these studies, knockout strains of individual ATG genes showed defects of starvation-induced autophagy. It is now widely accepted that at least 30 yeast genes are actively involved in autophagy. More than 50 additional yeast genes may be required for autophagy, which also play
key roles in other pathways (Klionsky et al., 2003). The precise cellular and biochemical function of these genes in the autophagic pathway remains to be elucidated. Although the role of autophagy in the aging process has yet to be fully defined, studies in model organisms (C. elegans) and mammals (mice) have begun to understand a growing number of aging processes that are influenced by autophagy. It is now largely accepted that autophagy affects several cellular activities crucial for longevity and healthy aging (eulongevity).

**Types of autophagy**

Eukaryotic cells are equipped with several degradation systems, predominantly two proteolytic systems contribute to cellular clearance: the ubiquitin-proteasome and the lysosomal systems. Autophagy is responsible for the degradation of whole organisms and structures (bacteria, viruses), whole organelles, as well as long-lived soluble proteins (Reggiori and Klionsky, 2002; Wang and Klionsky, 2003). The differences among the various autophagic mechanisms reside in the type of cargo/waste, the route and mechanism for its delivery to lysosomes and the physiological conditions in which each process is activated. Despite the variety of autophagic processes that have been described, they all fall into one of three main types: macroautophagy, microautophagy and chaperone-mediated autophagy (Mizushima et al., 2008, Klionsky, 2005; Cuervo, 2004).
Chaperone mediated autophagy

Most eukaryotic cells are equipped with form of autophagy, known as CMA (Dice, 2007). CMA is selective for a particular group of soluble cytosolic proteins that contain a specific sequence signature. Unlike autophagy, CMA does not require intermediate vesicle formation (Majeski and Dice, 2004). Once the specific signature sequence is identified, these soluble proteins are directly translocated to the lysosomal membrane. The specific signature sequence, which is present in all CMA substrates, is biochemically related to the pentapeptide KFERQ (Dice, 1990). All substrates with this sequence are then targeted to lysosomes. The cytosolic molecular chaperone heat shock protein 70 (hsp70) and its co-chaperones recognize the signature motif in substrate proteins (Hayes and Dice, 1996). The substrate/chaperone complex is then targeted to the lysosomal membrane, where it binds to a receptor protein, the lysosome-associated membrane protein type 2a (LAMP-2a) (Cuervo and Dice, 1996). A second chaperone, lysosomal hsc73 (lys-hsc73), is required for complete translocation of the substrate protein/hsc70 complex into the lysosomal matrix, where it is completely degraded by lysosomal proteases (Terlecky et al., 1992).

Microautophagy

The third form of autophagy is microautophagy (Klionsky, 2005). The characteristic feature of the microautophagy process is that the lysosomal membrane itself either invaginates or exvaginates to engulf an organelle. Unlike macroautophagy, there is no sequestering double-membrane formed in the cytoplasm (Mortimore et al., 1988). Pexophagy (Farre and Subramani, 2004) and Mitophagy
(Kim et al., 2007) involve the selective sequestration or engulfment of peroxisomes and mitochondria, respectively, and their delivery to lysosomes. The process of microautophagy is poorly characterized, at least in mammalian cells. Variations of microautophagy have been described in yeast for the selective degradation of peroxisomes (micropexophagy) or even nuclear regions (piecemeal microautophagy) (Klionsky et al., 2007).

**Macroautophagy**

Classic autophagy or macroautophagy is an inducible form of autophagy that becomes activated under stress conditions (nutrient deprivation, infections, and toxins) (Dice, 2000). Macroautophagy is the most extensively studied and quantitatively the most important form of autophagy. In this process, entire regions of the cytosol are sequestered by a *de novo*-synthesized membrane that seals into an autophagosome (Fig. 3) (Yorimitsu and Klionsky, 2005). These double-membrane vesicles fuse with secondary lysosomes, and in the process, acquire the proteases required for degradation of the sequestered material. Two major intracellular kinase complexes, the beclin-VPS34 complex and the mTOR (mammalian target of rapamycin, as it is referred in mammalian cells) complex, act in coordination to modulate formation, circularization, and fusion of the autophagic compartments with lysosomes (Yorimitsu and Klionsky, 2005; Ohsumi, 2001). For a long time, macroautophagy was considered as an inducible form of autophagy. Growing evidence though, indicate the existence of basal macroautophagy, essential for maintenance of cellular homeostasis in different organs (Hara et al., 2006, Komatsu
et al., 2006; Nakai et al., 2007). Here on, in the subsequent text autophagy refers to Macroautophagy.

**The (Macroautophagy) Autophagy Process**

The stages involved in the process of autophagy have not as yet been precisely delineated, especially for mammalian cells. Studies in the yeast *S. cerevisiae* have provided insight into the mechanisms involved and based on these studies, the following steps of autophagy are largely accepted: induction and cargo selection, vesicle nucleation and expansion, lysosome targeting, lysosome docking and autophagosome-lysosome fusion, vesicle breakdown and recycling (Klionsky and Emr, 2000).

Autophagy may be either a selective or nonselective process (Reggiori and Klionsky, 2005). In *S. Cerevisiae*, Cvt is a transport mechanism involved in the recognition and packaging of cargo and is considered a selective process. However, this pathway is limited only to yeast; it is not present in any other organism (Shintani and Klionsky, 2004). In general, macroautophagy is considered to be non-specific, however there are also specific type of macroautophagy such as in certain cellular conditions mitochondria are selectively trapped by autophagosomes. Further some types of microbes are selectively killed by autophagy. Growing line of evidence has revealed that there are reliable substrate for selective autophagy in mammals such as p62 and Nbr1 (Bjørkøy et al., 2005, Komatsu et al., 2007, Pankiv et al., 2007, Ichimura et al., 2008, Kirkin et al., 2009a; Kirkin et al., 2009b). SQSTM1/p62 is the first proposed example of receptor proteins that mediate recognition and targeting of
ubiquitinated substrates for their degradation via selective autophagy (Bjørkøy et al., 2005). Recently it has been shown that NBR1 (neighbor of BRCA1 gene 1) also act as receptors for selective autophagosomal degradation of ubiquitinated targets (Kirkin et al., 2009a). Further, it is proposed that NBR1 together with p62 promotes autophagic degradation of ubiquitinated targets and simultaneously regulates their aggregation when autophagy becomes limited (Kirkin et al., 2009b).

At the molecular level, the serine/threonine protein kinase TOR (target of rapamycin, as it is referred in yeast) is involved in the induction of autophagy (Petiot et al., 2000). Phosphorylated TOR is part of the induction complex and acts as a negative regulator of autophagy (Schmelzle and Hall, 2000). Phosphorylated TOR negatively controls autophagy, primarily by acting on the signaling cascade that controls general translation and transcription. In addition, activated TOR induces hyperphosphorylation of Atg13, which lowers its binding affinity to other Atg interacting proteins, thereby inhibiting autophagy (Kamada et al., 2000).

The initial formation of an autophagosomal membrane takes place by enwrapping the degradative cargo within a double membrane, which eventually elongates to form a vesicle called an autophagosome. The exact origin of autophagosomal membranes is controversial. Studies in mammalian cells have suggested that autophagosomal membranes originate from the ribosome-free region of the rough endoplasmic reticulum (Dunn, 1990). Alternatively, a poorly characterized organelle called a phagophore, has also been suggested as the origin of autophagosomes and other vesicular structures (Seglan et al., 1996). In yeast, a
unique perivacuolar structure, called PAS (preautophagosomal structure), has been proposed as the precursor of autophagosomes due to the transient association of several Atg proteins with it (Noda et al., 2002). A PAS-like structure has not yet been identified in any other endomembrane system (Kim et al., 2002).

Vesicles will elongate and extremities will fuse to complete the double-membraned structure. Phagophores elongate and completely encircle the cargo to form an “autophagosome” or “autophagic vacuole” (Fig. 3) (Reggiori and Klionsky, 2002). Most of the proteins involved in vesicle expansion and maturation steps are retrieved to the original pool because they do not associate with the complete and mature autophagosome (Levine and Klionsky, 2004, Reggiori and Klionsky, 2002; Klionsky, 2005). Therefore, it has been suggested that the proteins involved in autophagosome formation are retrieved for future use. Atg8 (LC3) is the exception; it is found on the mature autophagosome and can be used as a valuable marker to track these structures (Ohsumi, 2001).

The next critical step is transport and fusion of the autophagosome with the lysosomes. The outer membrane of the autophagosome completely fuses with the outer membrane of the lysosome to make a path for the inner membrane bound autophagic vacuole. The inner membrane and its enclosed cytoplasmic contents, which together are called an “autophagic body”, are released into the lumen of the lysosome (Fig. 3). The machinery required for the process of vesicle fusion includes SNARE proteins and the class C Vps/HOPS complex (Wang and Klionsky, 2003). The ultimate step in the autophagic process is degradation of the autophagic body.
content by lysosomal enzymes, which ensures recycling of essential cytoplasmic contents.

**Molecular Regulation of Macroautophagy**

Regulation of autophagosome biogenesis, maturation, and fusion with lysosomes is a complicated process and a number of diverse signaling complexes and pathways are involved (Fig. 2). Induction of autophagosome biogenesis requires two complexes. The first one, that initiates vesicle formation, contains the Class III PI3K (Vps34), Beclin1/Atg6, Atg14 and Vps15/p150. The second one, responsible for the vesicle nucleation, contains Atg1, Atg11, Atg13 and Atg17, the association of which is controlled by TOR. TOR is a classic serine/threonine kinase.
Fig.2 Regulation of autophagy: important pathways regulating autophagy includes TOR (target of rapamycin), which is at the center of the diverse pathways linked to autophagy, Class I and Class III PI3 kinase pathways.
Nutrient starvation or treatment of yeast cells with rapamycin (a specific inhibitor of TOR) rapidly induces an increase in autophagosome formation (Beck and Hall, 1999). Inhibition of TOR results in dephosphorylation of Tap (Klionsky et al., 2003) causing its dissociation from PP2A. PP2A becomes activated, causing dephosphorylation of downstream targets and activation of Atg1. In mammalian cells, mTOR appears to modulate autophagy in a manner similar to that observed in yeast. Likewise, the activity of the mammalian PP2A orthologue is strongly correlated to induction of autophagy (Holen et al., 1992).

The observation that 3-methyladenine (PI3K inhibitor) inhibits autophagy was the first evidence that implicated the Vps34/class III PI3K family of kinases in the process (Seglen and Gordon, 1982). In addition, other PI3K inhibitors, such as wortmannin and LY294002, have also been found to inhibit autophagy. In yeast, Vps34 is primarily involved in vacuolar protein targeting, through the endosomal/prevacuolar compartment, to form a complex with Vps15-Vps38-Atg6 (Kihara et al., 2001a). At the same time, Vps34 has been found to associate with Vps15-Atg6-Atg14 on pre-autophagosomal structure (PAS) or phagophore assembly site (Obara et al., 2006). PAS is a perivacuolar site where transient accumulation of most of the Atg proteins takes place and considers as autophagosomes origin site (Suzuki et al., 2001). Atg 14 and Atg 6 are important in mediating the localization of other Atg protein to the PAS (Suzuki et al., 2007). However, the mechanism by which Vps34 affects autophagosome formation is largely unknown. In mammalian cells, there are three classes of PI3Ks. So far, only Class I and Class III PI3K have been implicated in autophagy, primarily in the early steps of autophagosome formation.
(Petiot et al., 2000) Class III PI3K has been found to associate with beclin-1 and p150, which are the orthologues of yeast Atg6 and Vps15, respectively. Class III PI3K regulates diverse molecular pathways, including several involved in tumor formation.

The first autophagy-related tumor suppressor gene reported was Beclin-1, the mammalian orthologue of yeast Atg6, which showed a relationship between autophagy and cancer (Liang et al., 1999). Beclin 1 was identified as a new binding partner for Bcl-2 by Liang et al using yeast two-hybrid system (Liang et al., 1998) and later on Beclin 1 was the first protein shown to indispensable for autophagy (Liang et al., 1999). Beclin1/Vps34 complex produces Phosphatidylinositol-3-phosphate which is an early step in the autophagosome formation (Kihara et al., 2001b). This step is important for the recruitment of other ATG proteins. Afterwards, the sequential recruitment of ATG12 and its covalent binding with the ATG5 pool on the isolation membrane triggers autophagosome formation. Now, ATG12–ATG5 conjugate binds with ATG16 and this trimeric complex oligomerizes and localize on the external surface forming the autophagosome (Fig. 3). ATG12–ATG5–ATG16 and other ATG complex signals the recruitment of LC3 (ATG8) and forms ATG12–ATG5 and LC3 conjugate. Interaction of Beclin 1 with Vps34 and Vps15 is conserved in mammals (Kihara et al., 2001b). Additionally, Beclin 1 interacts with UVRAG (the UV irradiation resistance-associated gene protein) (Liang et al., 2006), Ambra1 (activating molecule in Beclin 1-regulated autophagy) (Fimia et al., 2007) and Bif-1 (also called endophilin B1) (Takahasi et al., 2007). UVRAG, Ambra1 and
Bif-1 are all positive regulator of autophagy and indispensable for the activation of autophagy, as well as for the optimal activation of Vps34.

**Fig.3 Process of Autophagy:** Autophagosome Biogenesis, Lysosomal fusion and interaction of Autophagy genes (ATG) at various steps. LC3 (ATG8) is well known autophagosome marker (LC3-II-PE).
In yeast, Atg1 is involved in both the Cvt and autophagic pathways, and it is found in association with Atg11 and Atg 13 (Kamada et al., 2000). Under nutrient-rich conditions, TOR activation causes hyperphosphorylation of Atg13 that prevents its association with Atg1 and it enables interaction of Atg1 with Atg11; this may determine the use of the autophagic machinery in the Cvt pathway. Under nutrient starvation or treatment with rapamycin, Atg13 becomes partially dephosphorylated, leading to an Atg1–Atg13 interaction, which subsequently triggers autophagy and generation of autophagosomes instead of Cvt vesicles. Atg17 is an Atg13 interacting protein that is part of the Atg1 complex and modulates the kinase activity of Atg1 (Kamada et al., 2000). Recently, hierarchy map analysis of Atg proteins involved in PAS organization has suggested that phosphorylated Atg 17 is the most basic protein in PAS organization: when it is specifically targeted to the plasma membrane, other Atg proteins are recruited to that location, suggesting that Atg17p acts as a scaffold protein to organize Atg proteins to the PAS (Suzuki et al., 2007).

In yeast, vesicle expansion and maturation is controlled by two ubiquitin like conjugation complexes; the Atg12–Atg5 conjugation complex and the Atg8 lipidation complex. In mammalian cells, the Atg8 orthologue MAP-LC3 (microtubule-associated protein light chain 3) is associated with GATE-16 (Golgi associated ATPase enhancer of 16 kDa) and GABARAP (γ-aminobutyric acid type A receptor-associated protein) (Okazaki et al., 2000). This complex was found to interact with ULK1, the mammalian orthologue of Atg1 (Yan et al., 1998). GATE-16 and GABARAP are the other two mammalian Atg8 orthologues. For vesicle expansion, the carboxy terminus of Atg8 (MAP-LC3) must first be removed by the action of the
cysteine protease Atg4. This cleavage exposes a reactive glycine in the C-terminus of Atg8 (MAP-LC3-I) that is subsequently activated by the Atg3 and Atg7 enzymes, and covalently linked to phosphatidylethanolamine (MAP-LC3-II-PE) on the autophagosomal membrane. This action of Atg3 and Atg7 requires activation by the Atg12–Atg5–Atg16 conjugation system, which dissociates from the membrane once autophagosome maturation is completed. The subsequent fusion with the lysosome (vacuole in yeast) leads to engulfment of the autophagosome, breakdown of its internal membrane and degradation of its cytosolic content by the action of lysosomal hydrolases.

**Nutrient regulation of Macroautophagy**

A host of dietary factors can influence various cellular processes and thereby potentially influence overall cancer risk and tumor behavior (Singletary and Milner, 2008). A wealth of evidence links diet habits and the accompanying nutritional status with cancer risk and tumor behavior (Popkin, 2007; Thomasset et al., 2007; Singletary et al., 2005). Characterizing how essential and nonessential dietary constituents can modulate single or multiple hallmark processes of carcinogenesis (Hanahan and Weinberg, 2000) is fundamental to identifying more effective mechanistic-based strategies for cancer prevention and therapy. To date, numerous structurally diverse biologically active food components have been reported to interfere with intercellular and intracellular signaling pathways that may activate critical cellular proto-oncogenes or inactivate tumor suppressor genes (Singletary et al., 2005; Hanahan and Weinberg, 2000). Consequently, these altered pathways can
modify growth autonomy, proliferation, angiogenesis, and metastasis and dysregulate cellular processes. Furthermore, a host of dietary factors can stimulate programmed cell death (Martin, 2006).

In this regard, it is now apparent that the demise of cancer cells not only can follow the well-characterized type I apoptotic pathway but also can proceed by stimulation of nonapoptotic modes such as type II (macroautophagy-related) and type III (necrosis) or combinations thereof (Ricci and Zong, 2006; Leist and Jaattela, 2001; Lockshin and Zakeri, 2004). However, in contrast to apoptosis, the induction of macroautophagy may contribute to either cell survival or death in response to a stressor. As described in more detail in above sections, autophagy is a degradative process by which cytoplasmic constituents of cells are engulfed within a cytoplasmic vacuole and delivered to the lysosome for degradation. Paradoxically, depending on the circumstances, this process of “self-consumption” may be involved in both health and disease progression (Shintani and Klionsky, 2004; Levine and Kroemer, 2008; Mizushima et al., 2008; Krysko et al., 2008; Eskelinen et al., 2005).

The canonical up-regulator of autophagy is nutrient deprivation and starvation (Kadowaki et al., 2006). Thus, autophagy is sensitive to levels of nutrients such as amino acids and glucose; hormones such as insulin and glucagon; and growth factors and cytokines such as insulin-like growth factor I, TNFα, and interleukin-3 (Tsujimoto and Shimizu, 2005). A coordinated response to nutrient (particularly amino acid) deprivation is regulated in mammals largely by cellular “nutrient sensors,” a major example of which is the target of rapamycin (TOR) kinase. The
mammalian TOR (mTOR) kinase is a repressor of autophagy and is an important controller of cell growth and proliferation. Therefore, it is not surprising that it is regulated by and communicates with multiple signaling pathways (Fingar and Blenis, 2004; Bhaskar and Hay, 2007; Sarbassov et al., 2005; Feng et al., 2005; Dann et al., 2007; Feng et al., 2007; Pattingre et al., 2008).

Another sensor of cellular bioenergetics, specifically in response to energy depletion, is the AMP-activated protein kinase (AMPK). AMPK is an activator of tuberous sclerosis complex 1/2, which, as a suppressor of mTOR, increases autophagy. Decreased energy availability leading to an increased intracellular AMP/ATP ratio results in activation of AMPK, in part, via the upstream kinase activities of the tumor suppressor LKB1 and Ca$^{2+}$/calmodulin kinase kinase β (Liang et al., 2007; Hoyer-Hansen and Jaattela, 2007; Hardie et al., 2007). There is also emerging evidence that other signal transduction pathways may regulate autophagy in a cell-specific manner. Thus nutrient status and metabolic stressors can influence the autophagic response via signaling networks that often, but not exclusively, converge at mTOR and that include both oncogene- and tumor suppressor gene–regulated intermediate steps.

**Functions of autophagy**

Autophagy is evolutionary conserved cellular catabolic process in eukaryotes which is required to maintain cellular homeostasis. Autophagy is responsible for the turnover of most long-lived proteins, macromolecules, protein aggregates, misfolded proteins and whole organelles. Autophagy occurs at a basal level in cells during
normal conditions and in important for maintaining intracellular housekeeping task which are require for cellular homeostasis. However, during stress, such as nutrient starvation, autophagy can also be induced as a way to produce nutrients for the cellular survival. So it helps cellular adaptation to nutrient deprivation. So in a normal cell autophagy regulates energy homeostasis and protein and organelle quality control. In multicellular eukaryotes autophagy is involved in plethora of functions (along with above described functions). Autophagy is required for protection against pathogens (intracellular virus and bacteria), a mean for antigen presentation, act as innate immune response and autophagy proteins play crucial role in immunity and inflammation. Autophagy also plays a crucial role in neonatal development, ageing, neurodegeneration, cell death (Excessive cannibalism of cells own constituents) and cancer. So in conclusion, autophagy can act as a cytoprotective mechanism to prevent various diseases, and dysfunctional autophagy leads to pathology.

Recent advances in the molecular characterization of autophagy have allowed the development of knockout and transgenic models that enable now to analyze the physiological role of autophagy. The classical role described for autophagy in the cellular adaptation to starvation has now been confirmed in the knock-out models for different essential autophagy genes (Kuma et al., 2004). Autophagy is thus required as a main source of both amino acids and energy in basal conditions as well as under stress. In this respect, macroautophagy and CMA seem to fulfill complementary functions. Thus, macroautophagy is maximally activated in different cell types during the first hours of starvation but it is gradually replaced by activation of CMA as starvation persists. Furthermore, models with altered autophagy have also revealed
novel functions for the autophagic system, most of them revolving around the ability of this pathway to eliminate damaged structures inside cells. This clearance has proven to be essential in maintenance of cellular homeostasis in many organs and tissues (Komatsu et al., 2005; Komatsu et al., 2006; Hara et al., 2006, Nakai et al., 2007). Different studies support an important role for CMA in the removal of altered proteins, (Cuervo, 2004; Massey et al., 2006; Wing et al., 1991; Cuervo and Dice, 1999) however, removal of damaged organelles and protein aggregates can only be attained via macroautophagy (Lemasters, 2005; Yorimitsu et al., 2006; Iwata et al., 2005; Rubinsztein et al., 2005).

Recently, a tight connection between autophagy and cell death has also been proposed. Although based on morphological descriptions, autophagy has been categorized as type II cell death (or non-apoptotic), the evidence supporting an active role of autophagy in cell death is still not clear (Debnath et al., 2005). In most conditions, macroautophagy has been shown to play a protective role against different types of stressors and thus act as a mechanism of cell defense rather than cell death, (Cuervo, 2004; Levine 2005; Levine 2007) and this seems to hold true for CMA (Massey et al., 2006) as well. It is only under conditions in which autophagy does not succeed in its attempts to protect cells against internal or external aggressors that the autophagic pathway itself fails and cellular death results. There are, however, instances in which uncontrolled activation of autophagy has been shown to result in cell death (Pattingre et al., 2005).

Autophagy, and in particular macroautophagy, has been shown to play a major role in processes involving major tissue or cell remodeling, such as
embryogenesis (Fimia et al., 2007; Qu et al., 2007; Tsukamoto et al., 2008). Furthermore, functional autophagy is essential in the cellular defense against pathogens (virus, bacteria, etc.). In fact, xenophagy was the term coined to refer to the engagement of autophagic machinery for the degradation of extracellular pathogens (Levine, 2005; Paludan et al., 2005). Both macroautophagy and CMA have also been shown to participate in the processing of exogenous antigens and autoantigens for presentation via MHC class II complexes to be destroyed by recognition via CD4+ T cells (DengJel et al., 2005; Zhou et al., 2005).

In conclusion, the different forms of autophagy participate as effectors or regulators of a growing number of cellular and organismal functions, reinforcing, thus, the physiological importance of this process. Also, various autophagy knockout and transgenic models that have provided invaluable information about the physiological functions of autophagy have also helped to establish connections between autophagy and different diseases.

**Autophagy in diseases**

Several human diseases are associated with decreased autophagic activity, particularly in nondividing cells of the nervous and muscle systems where turnover of intracellular proteins may be critical (Rajawat et. al., 2008). Muscular disorders, known as vacuolar myopathies, are associated with massive accumulation of imperfect autophagic or lysosomal vacuoles (Nishino, 2003). Danon disease is characterized by cardiomyopathy and mild mental retardation and is caused by a deficiency in LAMP-2, the transmembrane protein involved in endosomal and lysosomal fusion during the late maturation phase of the autophagosome (Nishino et
Accumulation of aberrant autophagosomes has also been associated with X-linked myopathy (X-MEA), inclusion body myositis and Marinesco-Sjögren syndrome (Kalimo et al., 1988; Nonaka, 1999; Goto et al., 1990).

Reduced autophagic activity is also associated with neurodegenerative disorders, such as Parkinson's (PD), Alzheimer's (AD), Huntington's (HD), and transmissible spongiform encephalopathy (prion disease) (Anglade et al., 1997; Cataldo et al., 1996; Kegel et al., 2000; Liberski, 2004). Altered activity of proteolytic systems and the occurrence of intracellular protein aggregates (inclusion bodies) are characteristic of these neurodegenerative diseases. The protein that accumulates in PD is mutated α-synuclein, in HD the protein is an abnormally expanded form of huntingtin, and in AD, protein deposits of overproduced β-amyloid precursors respectively (Stefanis et al., 2001; Venkataraman et al., 2004). Reduced autophagy has also been associated with chronic liver disease and hepatocellular carcinoma due to retention of mutant alpha (1)-Anti-Trypsin Z (ATZ) protein in the ER and mitochondria of hepatocytes (Teckman et al., 2004). One could hypothesize that perturbations at any stage of autophagosome biogenesis, maturation, or fusion with lysosomes would result in progressive accumulation of these aggregates and lead to disease progression.

In addition to the classic aging disorders and those of premature aging, autophagy has also been implicated in pathogenesis of infectious diseases. In general, autophagy functions as a protective mechanism against infections. Most foreign invaders (bacteria, parasites, viruses) must actually bypass the autophagic line of
defense before they can establish infections. In certain cases, some microorganisms have developed strategies to manipulate the autophagic machinery to their own advantage. After escaping the endophagocytic pathway and preventing fusion with lysosomes, the bacterium *Brucella abortus* shelters and replicates itself in autophagosome-like vesicles (Dorn, 2008). *Legionella pneumophila* secretes a product that activates autophagy and allows it to replicate itself directly inside autolysosomes (Coers, 2000). Likewise, a virulence protein, ICP34.5, in the *Herpes simplex* virus antagonizes the autophagic pathway and allows the virus to escape degradation (Tall et al., 2002). Recent immunological studies have also indicated the importance of autophagy in antigen presentation and successful cellular defense. It can be speculated that the decline in autophagic activity with age may contribute to the apparent increased risk of infectious disease in the elderly.

Since autophagic elimination of aggregated proteins and damaged organelles preserves cells from further damage, autophagy could serve a protective role. Enhancement of autophagic activity in aging organisms can have preventive or therapeutic potential. Caloric restriction-induced autophagy has a well-documented effect in extending life expectancy, at least in lower eukaryotic organisms and some laboratory mammals (Bergamini et al., 2004). The mTOR inhibitor and autophagy promoter, rapamycin, has shown promising anticancer activity in clinical trials (Guertin and Sabatini, 2005). Bacterial and viral proteins that modify autophagosome formation could also be harnessed to modulate this degradative pathway for therapeutic purposes.
Autophagy and neurodegeneration

Autophagic dysfunction has been described as one of the bases of pathogenesis in a growing number of neurodegenerative disorders. The main reason for this relationship is the fact that most neurodegenerative disorders can indeed be classified under the category of protein conformational disorders, a subset of diseases which result from the accumulation of misfolded proteins inside cells. Protein misfolding in these diseases can result from either mutations or post-translational modification by intra- and extracellular stressors. These misfolded proteins often organize into insoluble toxic oligomers and aggregates which accumulate inside cells as inclusion bodies.

Chaperones and the ubiquitin/proteasome system have been shown to be the first line of defense against these misbehaving proteins. In recent years, a role for CMA in the handling of altered proteins when still in a soluble form has also been demonstrated (Cuervo et al., 2004; Martinez-Vicente et al., 2008). Failure of any of these two defensive mechanisms leads to upregulation of macroautophagy, the only proteolytic pathway able to engulf and remove insoluble protein aggregates (Iwata et al., 2005; Ravilumar et al., 2002). Macroautophagy is thus responsible for the compensatory state in which cell viability is preserved through the activation of this pathway. However, as levels of misbehaving proteins continue to increase gradually, the constitutive activation of macroautophagy becomes insufficient to preserve a stable proteome. The decreased function of this autophagic system is often the consequence of direct blockage by misfolded or altered proteins but aggravating conditions such as aging and oxidative stress also contribute to autophagic failure.
The inability to maintain the autophagic response leads to accumulation of the altered proteins, partially undigested products, and cytosolic vesicles, which alter normal cellular function. Eventually, by mechanisms which are still unclear, these conditions result in cell death (Bandhyopadhyay and Cuervo, 2006).

Although the participation of autophagy in neurodegeneration requires more extensive investigation, the molecular basis for the autophagic dysfunction in some of these diseases has started to be elucidated. Thus, in the case of AD, the toxic protein product (A-beta) seems to interfere with the ability of lysosomes to clear up autophagic vacuoles formed in the cytosol of affected neurons. Upregulation of the endocytic-lysosomal system becomes evident in the affected brain regions at very early stages, which has been interpreted as a mechanism to cope with the fusion problem between autophagic vacuoles and lysosomes (Yu et al., 2005). As the disease progresses, lysosomal efficiency decreases. At the final stage, lysosomal leakage of proteolytic enzymes can be seen, which is detrimental for the affected neurons (Nixon, 2006; Nixon et al., 2000). The molecular basis for CMA failure in familial forms of PD has also been investigated. In this case, pathogenic forms of a-synuclein, one of the proteins that accumulate in the inclusion bodies of affected neurons, directly interfere with the ability of lysosomes to internalize cytosolic proteins via CMA. Mutant and post-translational forms of a-synuclein bind with abnormally high affinity to the CMA receptor at the lysosomal membrane, thus blocking this type of autophagy (Cuervo et al., 2004; Martinez-Vicente et al., 2008).
Autophagy and infectious diseases

The recently described role for autophagy in innate and adaptive immunity has led to the identification of a failure in proper autophagic function as the basis for some infectious diseases. Although certain intracellular pathogens are killed by autophagy, several others can use the autophagic compartments for their own benefit as sites for survival and replication. For example, after intracellular uptake, *Brucella abortus* and *Porphyromonas gingivalis* transit from early autophagosomes to late autophagosomes and prevent the formation of autolysosomes, either by delaying the autophagosome-lysosome fusion or by redirecting the normal autophagic trafficking. Survival of *P. gingivalis* through subversion of the host autophagic pathway can be viewed as a bacterial strategy to evade the innate immune system and persist in the host (Rodrigues et al., 2008). Another bacterium, *Legionella pneumophila*, seems to reach autolysosomes that already contain lytic enzymes and replicates there. The hydrolases of the host are, in this case, the ones processing the sequestered products to provide energy for the pathogen. An interesting finding in this case, is that autophagy is directly activated by a product secreted by the bacteria. If secretion in bacteria is blocked, autophagy is no longer stimulated and the bacteria are destroyed in phagolysosomes (Swanson and Isberg, 1995). Similar strategies are used by other bacterial pathogens and viruses (Levine and Deretic, 2007).

**Autophagy and Immunity**

Autophagy is considered as important arm of innate immune response. Autophagy is involved in the engulfment of intracellular viruses for their degradation in lysosomes, Regulation of inflammatory transcriptional responses, apoptotic corpse
clearance, regulation of inflammatory cytokine production, regulation of type 1 interferon production. Additionally, autophagy genes are involved in the generation of both innate and adaptive immune responses. Autophagy and/or autophagy genes also play a role in delivering ligands (VSV and Sendai virus) to TLRs for their activation. Activation of specific TLRs (TLR7 and TLR3) also initiates autophagy in some cells. Autophagy activation after TLR stimulation presumably functions to mediate xenophagy of entering pathogen. Up regulation of autophagy downstream of TLR stimulation may also enhance innate immune responses independently of xenophagy.

Autophagy participates in several aspects of immunity encompassing functions of the entire immune system to cell autonomous mechanisms. Autophagic mechanisms governing entire immune system function range from T cell development to generation of adaptive immune response. Indeed, autophagy within thymic epithelial cells is necessary for proper positive and negative selection of CD4 and CD8 T cell development (Nedjic et al, 2008). Furthermore, autophagy is required for CD4 T cell homeostasis. IL-7 stimulation of CD4 T cells provides important activation of mTOR resulting in inhibition of autophagy and cell proliferation. Lack of IL-7 signaling yields autophagy engagement and results in type II programmed cell death (Nedjic et al, 2008). Autophagy has also been implicated in the generation of an adaptive immune response. Essential to the development of an adaptive immune response is the ability for MHC class I and II to load and present antigenic peptides to CD8+ and CD4+ T cells (respectively). The MHC II machinery contained within lysosomes, loads antigenic peptides degraded in the late endosome/lysosome and is
then trafficked through the endocytic pathway to present MHC II: peptide on the surface of the cell for CD4+ T cell recognition. In certain cases, autophagy assists with deliverance of antigenic peptides to MHC II compartments to further CD4 T cell response (Kasai et al., 2009; Schmid et al., 2007; Paludan et al., 2005). Autophagy has also been reported to intersect with the cellular mechanism of cross-processing of MHC I antigen and suggested to be important for cross-presentation of infectious or tumor antigens (English et al., 2009; Li et al., 2008; Uhl et al., 2009). Together, these studies suggest that autophagy is necessary for generation of adaptive immunity and host protection; furthermore, autophagy aided antigen presentation may have implications for vaccine development (Lee et al., 2010; Jagannath et al., 2009).

Autophagy is under strict regulation to aid in defense against intracellular pathogens. Importantly, autophagy engagement must be a precisely regulated and controlled process in order to elicit immune function and avoid risk of autophagic cell death. Therefore, autophagy must undergo counter regulation to maintain a balance. Autophagy has been described as a mechanism that delivers several pathogens to lysosomes for killing. Indeed, autophagic degradation has been reported to target vacuolar and cytoplasmic pathogens including herpes simplex virus (HSV), human cytomegalovirus (HCMV), Group A Streptococcus, Mycobacterium tuberculosis, Listeria monocytogenes, Salmonella typhurium, Rickettsia conorii, Shigella Flexniri and Toxoplasma gondii (Jagannath et al., 2007; Birmingham et al., 2006; Nakagawa et al., 2004; Rich et al., 2003; Gutierrez et al., 2004; Ogawa et al., 2004).

Autophagy is triggered in response to both innate and adaptive stimuli. Antigen presenting cells like macrophages and dendritic cells undergo autophagy and
cytokine input or PRR stimulation can modulate this process. TLR ligands, LPS, imiquimod, and siRNA induce autophagy in macrophages to clear *M. tuberculosis* (Xu et al., 2007; Gutierrez et al., 2004). In the case of adaptive immunity, IFN-γ, and CD40 signaling triggers autophagy to sequester the vacuolar pathogens *M. tuberculosis* and *T. gondii*, respectively (Singh et al., 2006; Andrade et al., 2006). We have reported that CD40 stimulation on macrophages signals through its’ adaptor protein TNF Receptor Associated Factor 6 (TRAF6) to induce autophagosome formation around the parasitophorous vacuole of *T. gondii* (Andrade et al., 2006). Together, autophagy is capable of pathogen degradation induced by several stimuli to target a range of pathogens, both free within the cytosol and vacuolar pathogens that routinely evade host recognition. In addition to activation of autophagy, there is also a feedback loop in which autophagy stimulates TLR signaling. For example, autophagy delivers viral products of Sendai virus and vesicular stomatitis virus to TLR7 in endosomes and leads to activation and subsequent type 1 interferon production in plasmacytoid DCs (Heung et al., 2007).

**Pathogen Subversion of Autophagy**

Autophagy is considered as innate immune surveillance mechanism against intracellular pathogen including viruses. Selective autophagy which removes viruses and bacteria is known as Xenophagy. Autophagy can suppress replication of certain viruses and also involves in the presentation of some viruses to endosomal pattern recognition receptor which ultimately leads to immune system mediated clearance of viruses. Paradoxically,
viruses also exploit autophagy for their replication and for disease progression. Thus, autophagy may function as pro-viral or anti-viral mechanism. Several families of DNA and RNA viruses manipulate autophagy for their replication and survival.

Given the importance of autophagy as a powerful defense mechanism against intracellular pathogens, microbial virulence may be partly dependent on pathogen antagonism of this pathway. Indeed, there are a few pathogens reported to block autophagy induction through expression of specific proteins that interfere with Atg proteins and the formation of an isolation membrane. Shigella flexniri, an invasive bacteria, prevents Atg5 from targeting the Shigella protein VirG by secreting an additional protein, IcsB, which forms a complex with VirG to avoid Atg5 binding and autophagosome sequestration (Ogawa et al., 2004). Furthermore, Orvedahl et al. found that the neurovirulence protein Icp34.5 encoded by herpes simplex virus 1 (HSV-1) is able to bind to Beclin 1 and prevent autophagy induction (Orvedahl et al., 2007). Interestingly, mice that are infected with recombinant HSV-1, defective in Icp34.5, are severely deficient in neurovirulence, suggesting that autophagy is a potent host defense mechanism in vivo and HSV-1 must inhibit autophagy in order to result in a robust infection (Orvedahl et al., 2007).

It is possible that pathogens could modulate cell signaling pathways that activate or inhibit autophagy. As mentioned previously, Akt is an important signaling pathway involved in an upstream counter-regulatory cascade of autophagy. Several
pathogens utilize host proteins in order to promote survival. Akt is one such protein activated by a range of intracellular pathogens including *L. monocytogenes*, *S. typhurmium*, *M. tuberculosis*, and *T. gondii*. Interestingly, all of these pathogens are susceptible to autophagic degradation. It is unclear if pathogens are capable of modulating autophagy through disruption of host signaling cascades. Identification of pathogen subversion of autophagy and further determinants may reveal virulence factors and novel therapeutic targets.

**Autophagy and cancer**

Connections between autophagy and cancer are complex and, for the most part, still poorly understood. Most of the controversy originates as a result of a possible dual role of autophagy in the oncogenic process. In relation to cell death and cancer, autophagy is considered as both pro-survival mechanism as well as anti-survival or pro death mechanism. Autophagy acts as tumor suppressor by removing damaged organelles, cell growth control, reducing chromosome instability, endogenous antigen presentation, tumor immunity, physiological cell death, drug and radiation induced autophagic cell death. Paradoxically, autophagy is also convicted in the tumor progression or survival. Autophagy acts as cytoprotective mechanism that helps cancer cells to survive in conditions of low nutrient supply, resist anticancer treatment and prevention of apoptosis. So autophagy helps in cancer suppression as well as development.Inactivation of autophagy-specific genes, such as *beclin 1*, has been found in different types of tumors and this results in increased tumorigenesis in mice (Yue et al., 2003; Liang et al., 1999; Qu et al., 2003). In fact, overexpression of
beclin 1 to activate macroautophagy has been shown to effectively inhibit the formation of human breast tumors in mouse models, thereby supporting the notion of autophagy as a tumor-suppressor mechanism.

Autophagy also acts as tumor suppressor and decreased activity may contribute to development of tumors. Consistent with this theory, tumor-suppressor genes frequently mutated in cancer cells (e.g., p53, PTEN) are positive regulators of macroautophagy whereas, genes that are frequently activated in cancer, such as those encoding class I PI3K and AKT, downregulate macroautophagy (Mizushima et al., 2008; Ogier-Denis and Codogno, 2003). However, cancer cells have also been shown to utilize autophagy as a pro-survival mechanism in certain conditions such as the nutrient-limited environment in the core of solid tumors, or after oncogenic treatments aimed at producing damage to rapidly proliferating cells (i.e., ionizing radiation and chemotherapies) (Scarlatti et al., 2008; Paglin et al., 2001).

The complexity further increases, when taking into account cell type differences in the regulation and use of autophagy, thus suggesting that the role of autophagy in cancer biology may likely depend on the type, stage, and treatment conditions of the tumor. Pro-survival and pro-death functions of autophagy make cancer treatment more complicated. So targeting autophagy for cancer treatment will depend on pro or anticancer effects of autophagy in a given tumor condition. To kill a tumor cell turning autophagy on or off, either action may be correct. Many currently available antitumor agents are potent inducer of autophagy. Ultimately the question to whether to turn autophagy on or off may be resolved by information from animal experiments and clinical trials in cancer patients.
**Autophagy in Clinical Applications**

Current therapeutic targeting of autophagy in human disease is limited by an incomplete understanding of how the process contributes to pathogenesis, the lack of specificity of compounds that can influence autophagy, and the limited availability of candidate therapeutics with clinical efficacy (Van-Limbergen et al., 2009; Rubisztein et al., 2012; Rubisztein et al., 2007). Pharmacologic enhancement of autophagy (i.e. with vitamin D or adenosine 5′-monophosphate–activated protein kinase [AMPK] activators) promises to benefit certain diseases (i.e., infectious or neurodegenerative diseases) (Yuk et al., 2009; Høyer-Hansen et al., 2010; Yu et al., 2013). Sirolimus, a clinically approved immunosuppressive and anticancer drug that inhibits mTOR and thereby exerts pleiotropic effects, including the activation of autophagy, has been used to enhance autophagy in experimental models (Graziotto et al., 2012). Cytosolic or histone deacetylases (i.e., sirtuin-1, HDAC1, HDAC2, and HDAC6) may act as regulators of autophagic initiation and of autophagic flux (Moresi et al., 2012). Thus, HDAC inhibitors, or inhibitors of lysosomal acidification (e.g., chloroquine and hydroxychloroquine), may represent useful pharmacologic strategies for modulating autophagy. Current clinical trials are examining the usefulness of autophagy as a target in disease. Chloroquine and its derivative, hydroxychloroquine, are being tested for enhancement of chemotherapeutic efficacy (e.g., in the Preventing Invasive Breast Neoplasia with Chloroquine trial [ClinicalTrials.gov number, NCT01023477] and the Phase 1 Trial of MK-2206 and Hydroxychloroquine in Solid Tumors and Prostate Cancer [NCT01480154]), including therapies for ductal carcinoma (chloroquine) and for pancreatic adenocarcinoma, breast cancer, and non–small-cell lung cancer.
(hydroxychloroquine). The design of therapeutic agents is complicated by the fact that many autophagy proteins, as well as pharmacologic inhibitors (e.g., chloroquine), may also affect biologic processes independently of autophagy activation.

An improved understanding of the mechanism (or mechanisms) by which autophagy can prevent pathogenesis may lead to the identification of new targets for both diagnostic and therapeutic approaches. Drug screening for agonists or antagonists of autophagic activity, including upstream regulators and downstream targets of autophagy, may yield additional therapeutic targets. If the advances in autophagy continue at an accelerated pace, agents acting on autophagy may eventually provide useful therapies for human diseases.
Vitamin A/Retinoids

Vitamin A is a fat-soluble co-factor present in food that is essential for life. It plays an important role as a coenzyme in vital processes that the body needs to function normally. The structure of Vitamin A makes it good molecule for trapping light energy. It is the molecule responsible for transferring light energy into a chemical nerve impulse in the eye (Amann, et. al., 2011). It is also important for the regulation of biological activities such as embryogenesis, cell growth, differentiation, reproduction, apoptosis, and bone formation (Niles, 2000; Sun and Lotan, 2002; Mamede, et. al., 2011).

Physiological metabolites of Vitamin A are collectively known as retinoids. Although the most common dietary form of Vitamin A is retinol, there are over 4000 natural and synthetic molecules that are structurally and/or functionally related to this compound. Vitamin A is only obtained through the diet in the form of retinol, retinyl esters, or provitamin A (β-carotene). It is found in foods of plant origin such as sweet potatoes and carrots as β-carotene and in animal products such as liver and eggs as retinyl esters (Amann, et. al., 2011).

Retinol is either stored in the liver as retinyl esters or circulates in the bloodstream complexed with plasma retinol-binding protein (RBP) or transthyretin (pre-albumin). Plasma membrane receptors for the retinol-binding protein mediate the cellular uptake of retinol. After retinol has been taken up by the cell, it becomes bound to cellular retinol-binding protein (CRBP). Retinol is first converted to retinal, before being metabolized into all-trans retinoic acid (atRA) by a retinal dehydrogenase. The major physiologically active metabolite of retinol, retinoic acid,
becomes bound to cellular retinoic acid-binding protein (CRAPB). The CRABP bound retinoic acid is then delivered to the nucleus of the cell by CRABPII, where it will bind to and activate the retinoic acid receptor (Niles, 1998).

A)

![Chemical structure of retinol](image)

B)

![Chemical structure of all-trans retinoic acid](image)

C)

![Chemical structure of beta-carotene](image)

**Fig. 4** Chemical structures of Vitamin A Metabolites: A) retinol, B) all-trans retinoic acid, and C) beta-carotene.
Retinoic Acid Regulated Gene Expression

Retinoids signals their cellular effects on biological processes through the regulation of gene expression, which is due to the ability of atRA to bind and activate specific nuclear receptors. Various scientific studies have highlighted key regulators of the retinoid signaling pathway. There are two distinct sub-families of nuclear retinoid receptors. The first are the RARs (retinoic acid receptors) that bind all-trans retinoic acid and 9-cis retinoic acid. The RXRs (retinoid X receptors) bind only 9-cis retinoic acid. There are three distinct subtypes of RXRs and RARs (α, β and γ), which are encoded by different and highly conserved genes.

The RARs share functional domains common to other members of the steroid hormone receptor gene family. The A/B region located in the N-terminal portion functions as a ligand-independent transcriptional activation domain. The C region contains the DNA-binding domain that consists of two zinc-binding, cysteine-rich segments. The D domain acts as a hinge, whereas the E segment acts as a dimerization interface for ligand-dependent transcriptional activation. This region is highly conserved between all of the RAR subtypes. The C-terminal F region is different between the RAR subtypes and at the present time its function is not known. However, it is thought that it may bind the coactivator CBP/p300 (De Lera et. al., 2007; Niles, 1998).

![Fig.5 RAR domain structure and Function: A/B) Ligand -independent transcriptional activation domain, C) DNA binding domain (DBD), D) Hinge](image)

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domain, E) Ligand binding domain (LBD), dimerization domain and silencing domain, F) Domain with Unknown function

Retinoic acid induces phenotypic changes in target cells by inducing the transcriptional activity of the nuclear retinoid receptors. It is the main activator of RARs whereas the RXR serves as its common heterodimeric partner. The RXRs have the ability to also heterodimerize with other nuclear receptors. These include the thyroid hormone receptor (TR), Vitamin D receptor (VDR), PPARs (peroxisome-proliferator activated receptors), and numerous orphan receptors. The RAR-RXR heterodimer binds to a specific sequence located in the promoter region of the DNA called a retinoic acid response element (RARE) with a half site sequence similar to AGGTCA. It is a direct repeat (DR) of half sites with a spacer of 2 to 5 nucleotides (DR2-DR5). In the absence of ligand, the RAR-RXR dimer is bound to the RARE, and held inactive by co-repressors, such as SMRT (silencing mediator of retinoid and thyroid signaling) and N-CoR (nuclear receptor co-repressor). These co-repressors bind to the non-liganded receptors resulting in suppression of basal transcriptional activity. The ability of atRA to bind to the RARs results in destabilization of this complex and a conformational change in the receptors that allow for the disassociation of co-repressors and binding of co-activators, such as SRC-1 (steroid receptor co-activator 1) and CBP/p300. Consequently, atRA exerts its effects through changing the structure of the receptor, which allows for control of distinct molecular events at retinoid regulated promoters (Niles, 1998).

An important regulatory mechanism that co-activators and co-repressors have is that they harbor histone acetylation/deacetylation enzyme activity. Acetylation of lysine residues on nucleosome tails results in the relaxation of the chromatin
structure, allowing access of transcription factors to DNA and therefore, an increase in transcriptional activity. Co-activators, such as CBP/p300, contain histone acetyl-transferase (HAT) activity while co-repressors recruit enzymes that have histone deacetylase (HDAC) activity. The level of acetylation is controlled by these two opposing families of enzymes, and ultimately, the activity of the RAR/RXR complex.

**Functions of Retinoids**

Retinoids (vitamin A) are crucial for most forms of life. In vertebrates, they play vital roles in the developing nervous system and notochord and many other embryonic structures, as well as in maintenance of epithelial surfaces, immune competence, and reproduction (Fig. 6). The ability of all-trans retinoic acid to regulate expression of several hundred genes through binding to nuclear transcription factors is believed to mediate most of these functions. Retinoids have been called the master regulators of differentiation and play critical roles in development (McCaffery and Drager, 2000). Retinoic acid is essential for both embryonic and adult growth. Retinoids control patterning of the central nervous system influencing development of the hindbrain and spinal cord; the development of the cardiovascular system; development of the kidney, eye, ear; and the olfactory pathway, among others (Glover, et al., 2006; Hyatt and Dowling, 1997; LaMantia et al, 2000; Maden, 2006; Mendelsohn et al, 1999; Mollard et al, 2000; Romand et al, 2006; Vermot et al, 2003). In short, retinoids control the processes of proliferation, differentiation and apoptosis throughout an organism’s life. Several studies indicate that the effects of retinoids on cell proliferation, differentiation and apoptosis are retinoid-specific and cell-type specific.
Resinoids involvement in various functions: Resinoids have diverse physiological functions and very vital for the process of vision, development and differentiation. Resinoids induces apoptotic cell death by TRAIL receptors but role of resinoids in autophagy is not known.

**Retinoids in Cellular Proliferation**

Progression through the cell cycle, from the Gap1 (or growth phase 1) to mitosis (M), is tightly regulated by the levels and activity of specialized groups of proteins known as cyclins, cyclin-dependent kinases (CDK) and cyclin-dependent kinase inhibitors (CDKI) (Golias et al, 2004). Resinoids influence each one of these groups of proteins. For example, retinoic acid induces growth arrest in myeloid cell lines by up-regulation of p21/CIP1 and p27/KIP1 (CDKIs); down-regulation of cyclin E and cyclin D1/D3, cyclin A and cyclin B; decreased CDK activity; and de-phosphorylation of pRb (Dimberg and Oberg, 2003). In EBV-immortalized B lymphocytes, ATRA-, 9-cis RA- and 13-cis RA- triggered growth arrest is associated with multiple changes in G1 regulatory proteins including decreased activity of
CDK2, CDK4 and CDK6; decreased levels of cyclin D3 and cyclin A; as well as increased expression of p27/KIP1 (Zancai et al, 1998). Within the sebaceous gland, 13-cis RA has been shown to decrease proliferation of sebocytes as evidenced by decreased 3H-thymidine labeling after 12-weeks of treatment when compared to baseline (Landthaler, et al., 1980). No studies to date have examined the nature of this decrease in proliferation.

**Retinoids in Cellular Differentiation**

Differentiation can be defined as a series of biochemical and structural changes by which cells become specialized in form and function. The most studied example of retinoids controlling differentiation is the patterning and formation of the hindbrain during embryonic development (Glover, et al., 2006). In this case, the timing of and the gradient expression of retinoic acid determines the antero-posterior as well as the dorso-ventral axes of the hindbrain (Avantaggiato et al, 1996; Glover, et al., 2006). Excess retinoic acid in the anterior region results in a “posteriorizing” of the hindbrain whereas decreased retinoic acid in the posterior region results in an “anteriorizing” of the hindbrain (Glover, et al., 2006; Thompson et al, 1969), which upsets the normal developmental patterns leading to deleterious defects. In addition, the gradient of retinoic acid expression influences the expression of the *Hox* genes, which are critical in rhombomere segment determination (Simeone et al, 1990; Wilkinson et al., 1989; Wood et al, 1994). Furthermore, the pattern formation of the hindbrain is specific to the type of retinoid (i.e. all-trans retinoic acid (ATRA)). Exogenous administration of 9-cis RA in zebrafish has a more pronounced effect than ATRA in “posteriorizing” the hindbrain (Zhang et al, 1996). This central nervous
system example illustrates the fact that the amount of retinoic acid and the specific isoform of retinoic acid present can have profound effects on differentiation.

Retinoids also influence differentiation in skin. The earliest studies noted that deficiency of vitamin A in the diet of laboratory animals led to changes in the normal epithelium, with specific loss of the mucous secretory epithelium, while replenishing the vitamin A restored the normal phenotype (Wolbach and Howe, 1925). Retinoids inhibit the differentiation of keratinocytes as evidenced by decreased keratin 1, keratin 10, transglutaminase, loricrin, and filaggrin expression (Fisher and Voorhees, 1996). De Luca et al. have studied the effects of retinoids in mouse endocervical epithelia undergoing squamous metaplasia as a result of retinoid deficiency. Their studies show that vitamin A deficiency causes a simple-columnar epithelium to gradually become squamous metaplasia and that vitamin A concentration is a factor in maintaining a simple or more stratified epithelial morphology (De Luca et al., 1995). In addition to retinoid effects on epithelium, it can also influence the dermis. For example, it modulates the expression of the genes for hyaluronate and collagen, two major constituents of the dermis. It increases their expression, synthesis and concentration in the skin, helping to reduce wrinkle formation (Sorg et al, 2005). Within the sebaceous gland, retinoids inhibit differentiation as determined by decreases in sebum secretion with 13-cis RA treatment (Strauss, et al., 1980).

**Retinoids and Immune functions**

Studies over the years have demonstrated the pleiotropic influence of vitamin A on immunological fitness (Hall et al., 2011). Vitamin A elicits a broad array of immune responses through its metabolite, retinoic acid (RA). (Acin-Perez et al.,
Recent evidence indicates that loss of RA leads to impaired immunity, whereas excess RA can potentially promote inflammatory disorders. Vitamin A’s critical contribution to immunological health is shown by the fact that its supplementation dramatically curbs young-childhood mortality in endemic regions of malnutrition (Rahmathullah et al., 1990; Sommer, 2008; Sommer et al., 1986). Over the last 20 years it has become clear that retinoic acid influences multiple immune cell lineages and an array of immunological functions (Cantorna et al., 1995; Chun et al., 1992).

In populations where vitamin A availability from food is low, infectious diseases can precipitate vitamin A deficiency by decreasing intake, decreasing absorption, and increasing excretion. Infectious diseases that induce the acute-phase response also impair the assessment of vitamin A status by transiently depressing serum retinol concentrations. Vitamin A deficiency impairs innate immunity by impeding normal regeneration of mucosal barriers damaged by infection, and by diminishing the function of neutrophils, macrophages, and natural killer cells. Vitamin A is also required for adaptive immunity and plays a role in the development of T helper (Th) cells and B-cells. In particular, vitamin A deficiency diminishes antibody-mediated responses directed by Th2 cells, although some aspects of Th1-mediated immunity are also diminished. These changes in mucosal epithelial regeneration and immune function presumably account for the increased mortality seen in vitamin A-deficient infants, young children, and pregnant women in many areas of the world today (Stephensen, 2001). Historically, vitamin A deprivation and repletion and RA agonists have been shown to profoundly affect immunity. Retinoic
acid (RA) controls both the homing and differentiation of regulatory T cells (T(reg)) (Pino-Lagos et al, 2008). RA also regulate the antibody response in vivo. RA regulated factors known to be required for Immunoglobulin class switch recombination and modulated the population dynamics of B cells (Chen and Ross, 2005)

**Retinoids and Apoptosis**

On the basis of morphological characteristics, apoptosis has been defined as highly-regulated, well-orchestrated series of events culminating in nuclear condensation, DNA fragmentation, membrane-blebbing, cell shrinkage, and eventually phagocytosis of the dying cell (Wyllie et al, 1980). There are two well characterized pathways which lead to apoptosis: death receptor (extrinsic) and mitochondrial (intrinsic) apoptotic pathways. Death receptors on the cell’s surface detect extracellular stimuli and upon binding of their respective ligands, rapidly activate an intracellular caspase signaling cascade that results in apoptosis. The ligands for death receptors include tumor necrosis factor related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL, CD95L) (Slee et al, 1999; Smith et al, 2003; Wehrli et al, 2000). The mitochondrial apoptotic pathway is activated by intracellular damage sensed by the mitochondria itself resulting in permeability of the outer mitochondrial membrane and release of cytochrome c. (Green and Kroemer, 2005; Lucken-Ardjomande and Martinou, 2005). These two pathways converge with activation of caspase 3 and, in some cells, by activation of the protein Bid, a Bcl-2 family member.
It is well established that retinoids induce apoptosis in numerous cell types, both normal cells and tumor cell lines. 13-\textit{cis} RA reduces the survival and genesis of murine hippocampal neurons \textit{in vivo} (Crandall et al, 2004; Sakai et al, 2004). ATRA has been shown to induce apoptosis in primary and metastatic melanoma cells as well as inducing growth arrest followed by apoptosis in orbital fibroblasts isolated from Graves’ disease patients (Pasquali et al, 2003; Zhang and Rosdahl, 2004). In leukemia cells, 9-\textit{cis} RA inhibited cell growth and induced apoptosis to a greater extent than 13-\textit{cis} RA or ATRA; however, in adult T cell leukemia cells, all three retinoids were equally effective (Fujimura et al, 2003; Koistinen et al, 2002). These studies are only a few of many in the literature that demonstrate that the actions of retinoids are unique and specific to the model used. Natural and synthetic retinoids induce apoptosis by activation of the extrinsic or intrinsic pathways or simultaneous activation of both pathways in a retinoid- and cell-type specific manner. The cellular targets of retinoids leading to activation of the cell death pathways are just beginning to be revealed.

\textbf{Retinoids and Cancer}

Role of retinoids in cancer prevention was first suggested in 1925 when vitamin A was reported as required for epithelial cell homeostasis [Wolbach SB, Howe PR]. Since then various retinoid compounds have been characterized and used as both chemopreventative and chemotherapeutic agents for the treatment of many types of cancers. The vitamin A metabolite, retinoic acid (RA), first received attention as an interventional therapy upon discovery that it could substitute for more toxic chemotherapeutic regimens to dramatically improve the prognosis of acute
promyelocytic leukemia (APL), a malignancy caused by genetic translocations with the retinoic acid receptor (RAR), RARα (de Thé and Chen, 2010). Numerous investigations of APL have highlighted the ability of RA to promote myeloid cell differentiation (Kastner et al., 2001).

Derivatives of vitamin A, the retinoids, have reported activity in treating specific premalignant lesions and reducing incidence of second primary tumors in patients with prior head and neck, lung or liver cancers. Studies using Vitamin A deficient animals showed that there was a strong connection between deficiency and the susceptibility to cancer (Chambon, 1994; Sporn et al., 1976). The addition of retinoic acid to the media of various types of cancer cells grown in culture was able to significantly reduce proliferation (Niles, 2000; Bushue et al., 2010). Retinoic acid can induce differentiation in tumor cells such as neuroblastoma (Sidell, 1982), melanoma (Wood et al., 1990; Fligiel et al., 1992; Jacob et al., 1998; Niles, 2003), and promyelocytic leukemia (Breitman, et al., 1980). Retinoids are able to elicit their effects on malignant cell growth through the regulation of cell growth, differentiation, and apoptosis (Lotan, 1995; Bushue et al., 2010; Mamede et al., 2011).

There is evidence that demonstrates a connection between cellular transformation and altered expression of RARs. It is believed that the RAR-β2 receptor is critical for mediating the growth inhibitory effects of retinoids and its down-regulation is correlated with the loss of aTRA’s ability to inhibit cell growth (Lotan, 1996; Sun, 2004; Pavan et al., 2006; Bushue et al., 2010). The expression of this receptor is down-regulated in head and neck cancer, melanoma, breast, and oral cancers (Lotan, 1995; Fan et al., 2010). The reactivation of RAR-β2 expression leads
to reacquisition of atRA’s ability to inhibit cell growth (Xu, 2007), therefore it has been considered as a tumor suppressor gene. The silencing of RAR-β2 expression is due to epigenetic changes, mainly DNA methylation (Virmani et al., 2001; Shaw et al., 2006). Current research indicates that, while DNA methylation appears to be the predominant mechanism for silencing of RAR-β2 expression, there are other mechanisms by which this occurs, such as modulating the levels of acetylation of histones H3 and H4 (Fan et al., 2010).

Retinoids are vitamin A derivatives that critically regulate several physiological and pathological processes, including immune functions and cancer development. Accumulating evidence indicates that retinoids may exert beneficial effects in both immune-mediated disorders and tumors. With regard to cancer, retinoids directly target neoplastic cells by inducing differentiation, inhibiting cell growth or promoting survival. However, the efficacy of these compounds in cancer treatment probably resides in their ability to modulate also the function of immune effectors. Vitamin A derivatives are currently used in the therapy of acute promyelocytic leukemia and of cutaneous T cell lymphomas, but they could be effective also on B-cell malignancies. Clinical trials are ongoing to test their efficacy in solid tumors (Montrone et al., 2009)
Cation-independent Mannose-6-Phosphate receptor (CI-MPR)

Retinoic acid (RA) exerts diverse biological effects in the control of cell growth in embryonic development and oncogenesis (De Luca, 1991; Sporn et al., 1994). Several of these specific physiological functions or the non specific side effects of the retinoids cannot be attributed to the classical nuclear receptor i.e. RAR and RXR. Evidence suggests that other retinoid response pathways that are independent of the nuclear receptors may exist (Ahuja et al., 1997; O’Connell et al., 1996; Delia et al., 1993). It has been shown that the retinoid-induced apoptosis or growth inhibition is independent of the action of the nuclear receptors which suggests that other retinoid-response pathways may exist (Ahuja et al., 1997; Shao et al., 1995; O’Connell et al., 1996; Sun et al., 1997; Toma et al., 1998; Mologni et al., 1999; Clifford et al., 1999; Kim et al., 1995). Thus, despite our knowledge of the nuclear receptors for RA, how RA can exert a great diversity of biological effects is still not fully understood.

Interestingly, photoaffinity labeling studies has shown direct binding of ATRA to the cation-independent mannose-6-phosphate/IGFII receptor (CIMPR) with high affinity, however, the binding site for ATRA is not known (Kang et al., 1997). Photoaffinity labeling studies have demonstrated that the Mannose-6-Phosphate/Insulin Growth Factor-2 Receptor (M6P/IGF2R) is a novel receptor for Retinoic acid (RA) (Kang et al., 1997). RA binds to the M6P/IGF2R with high affinity ($K_D$ of 2–3 nM). The binding site for RA on the receptor protein is distinct from the binding sites for M6P and IGF2. Binding of RA enhances binding of the other ligands (e.g., M6P or M6P-containing proteins) to the receptor. In addition,
binding of RA to the M6P/IGF2R seems to facilitate the endocytosis and sorting functions of the receptor (Kang et al., 1998). Furthermore, another study showed that the interaction of RA with the M6P/IGF2R leads to an intracellular redistribution of the receptor and lysosomal enzymes (Kang et al., 1998). These studies suggested that RA may act as a functional promoter of the M6P/IGF2R by directly binding to its unique site on the receptor. Furthermore, in continuation of these studies, it was also demonstrated that M6P/IGF2R mediates the growth inhibitory effects of retinoids (Kang et al., 1999).

CI-MPR is a ubiquitously and constitutively expressed glycoprotein that plays fundamental role in endocytosis and degradation of extracellular ligands (IGF-II, uPAR), lysosomal enzyme trafficking and regulation of apoptotic and mitogenic effects (El-shewy et al., 2009). The primary function of CI-MPR is to sort and transport mannose-6-phosphate (M6P) bearing glycoproteins (such as hydrolases) from TGN to endosomes/lysosomes. However it is distinct from those for M6P and IGF-II on the receptor (El-shewy et al., 2009).

The cation independent mannose-6-phosphate receptor (CI-MPR) /insulin like growth factor 2 receptor (IGF2R) is a multiple ligand-binding cell surface receptor, ubiquitously expressed in human tissues. The CI-MPR is a large 300KDa glycoprotein which binds Mannose -6-phosphate (M6P), containing ligand and non-M6P ligands. The human CI-MPR is 2491 amino acid long and includes a N-terminal signal sequence of 40 amino acid, an extra cytoplasmic domain consisting of 15 homologous repeat sequences of 134-167 amino acid (2264 amino acid for the full extra cytoplasmic domain), a transmembrane domain of 23 amino acids and a
cytoplasmic domain of 164 amino acid which constitute the C-terminal domain. The cytoplasmic domain contains various sorting signals as well as various motifs to interact with adaptor proteins (Gosh et al., 2003).

**Functions of CI-MPR/IGF2R**

CI-MPR have myriad of important physiological functions. Approximately 90% of CIMPR molecules are located in the Golgi and endosomal compartments. Receptors (5-10%) are also present on the plasma membrane; these two cellular pools cycle continuously internalizing extracellular peptides and participating in Golgi to endosome transfer of M6P proteins (principally lysosomal enzymes) (Kornfeld, 1992). The major role of the CI-MPR is to facilitate the trafficking of lysosomal enzymes between TGN, endosomes and lysosomes. The CI-MPR is also rapidly internalized upon binding of other ligands including IGF2, granzyme B, UPAR/plasminogen, glycosylated Leukemia inhibitory factor (LIF), Angiogenic peptide proliferin, prorenin and Retinoids (Hawkes and Kar, 2004). The regulation motif “YSKV”, which binds assembly protein-1 and protein-2 (AP-1 & AP-2) is the main mediator of IGF2 R internalization via clatherin-coated vesicles (CCVS) and lies in the cytoplasmic tail.

Accumulating evidence suggests critical role played by CI-MPR in growth and differentiation. Binding of IGF-II to the CI-MPR results in the internalization and subsequent degradation of the ligand in the lysosomes, thereby suppressing mitogenesis by reducing IGF-II availability for binding to the IGF-I receptor. CI-MPR binds with the latent transforming growth factor- β (TGF- β), permitting cleavage into active form, which is a potent growth inhibitor for most cell types.
(Dennis and Rifkin, 1991). Retinoic Acid (RA) has been shown to increase lysosomal enzyme sorting via CI-MPR and stimulate IGF2R mediated IGF2 internalization, which contributes to the regulation of cellular proliferation by retinoids.

The CI-MPR gene is regarded as a tumor suppressor gene. The ability of CI-MPR to modulate local levels of mitogen IGF2 to facilitate activation of growth inhibitor TGF-β1 and to regulate trafficking of lysosomal enzymes indicates that it could be a tumor suppressor. Loss of CI-MPR function is associated with progression of tumorigenesis. Frequent loss of heterozygocity (LOH) at the CI-MPR locus has been reported in human liver, breast, lungs, ovarian and adrenocortical cancers. CI-MPR may be involved in the process of cell migration and invasion by IGF2 stimulation (Gosh et al., 2003).
Acidification of intracellular organelles

The luminal pH of endocytic organelles and lysosomes is acidic, and acidification and its regulation constitute an important part of endosome maturation (Huotari and Helenius, 2011). Early Endosomes (EE) have a pH in the 6.1–6.8 range, Late Endosomes (LE) in the 6.0–4.8 range, and in lysosomes the pH can drop to values around 4.5 (Maxfield and Yamashiro, 1987). The low pH not only provides a better environment for hydrolytic reactions, but it is also essential for membrane trafficking, for the sorting and routing of cargo, for the inactivation of internalized pathogens, etc. The difference in extracellular and EE pH (or between the TGN and LEs) provides the asymmetry needed to allow receptors to bind ligands in one compartment and release them in the other (Huotari and Helenius, 2011). Thus, the progressively decreasing pH in the endocytic pathway can provide incoming cargo a ‘sense’ as to their location within the pathway. This is most clearly illustrated by viruses and bacterial toxins that have a relative sharp pH threshold adjusted so that they are specifically activated when they arrive in EEs, in LEs, or in lysosomes (Mercer et al., 2010).

Acidification of various organelles requires many proteins, enzymes and proton pumps including the vacuolar H+-ATPase. The vacuolar H+-ATPase is an universal component of eukaryotic organisms. It is present on membranes of many organelles, such as endosomes, lysosomes, phagosomes, the Golgi apparatus and secretory granules, where its proton-pumping action creates the low intravacuolar pH found (Finbow and Harrison, 1997). The vacuolar H+-ATPase is a member of a family of
multi-subunit proton pumps. It is composed of a membrane sector and a cytosolic catalytic sector. It pumps protons from the cytoplasm to the lumen of the vacuole using the energy released by ATP hydrolysis. So, it is part of the cellular machinery that regulates the cytosolic pH. This pump is electrogenic, i.e. the movement of a proton results in the translocation of net charge that is not directly coupled to the transport of another ion to maintain electroneutrality, thereby creating an electrical potential difference across the membrane (Finbow and Harrison, 1997).

**Acidification of endosomes**

Acidification of endosomal compartments, its regulation and its roles in endocytosis have been extensively studied (Mukherjee et al., 1997; Clague, 1998). Extensive characterization of endosomal compartment pH using endocytosed fluorophores such as fluorescein has been carried out in Chinese Hamster Ovary cells (CHO cells) and in other fibroblasts. In CHO cells, sorting endosomes have a pH of 5.9-6.0, the recycling compartment of 6.4-6.5, whereas late endosomes have a pH below 6.0 and lysosomes are even more acidic, with a pH between 5.0 and 5.5. Endosomal acidity is maintained by an ATP-dependent proton pump. Various mechanisms may account for different acidities in different endosomal compartments. In some cells, acidification of early endosomes is counteracted by the electrogenic Na+/K+ pump. This pump generates an inside positive membrane potential that makes it more difficult to pump protons inside the endosome. This pump, which recycles back to the plasma membrane from early endosomal compartments, is present on early endosomes but not on late endosomes, thus allowing the late endosomal compartment to acidify to a greater extent. Chloride conductance also
regulates the pH of endosomes and lysosomes. It has been suggested that changes in membrane potential could affect the steady-state pH of an endosome by implying changes in the coupling between proton translocation and ATP hydrolysis by the vacuolar proton pump as the proton concentration and/or positive charge in the compartment builds up.

Acidification is an essential property for endocytic sorting and activity of lysosomal enzymes. The acidic environment is used to dissociate many ligands, including insulin, EGF, LDL from their receptors within sorting endosomes. This could result from a pH-dependent conformational change in the receptors. Another example of pH-dependent conformational change is the release of iron from Tf in the sorting endosomes, while apo-Tf remains bound to its receptor in the acidic endosomes. Some ligands require a lower pH than that found in the early endosomes to be released from their receptors; it is the case for MP ligands that are released from the CI-MPR at about pH 5.8, corresponding to the pH found in the late endosomes. The low pH of late endosomes and lysosomes also ensure the optimal environment for the activity of lysosomal enzymes that have acidic pH optima.

In addition, the control of the internal pH of endosomal compartments plays a role in membrane traffic. First, agents that neutralize endosomal pH such as ammonium chloride or monensin inhibit the transport of the fluid-phase endocytic tracer HRP between early and late endosomes (Sullivan et al, 1987; Cupers et al, 1997). Second, upon inactivation of the vacuolar H+-ATPase by bafilomycin A1, trafficking between early and late endosomes continues, but that to lysosomes is blocked, indicating that transport from late endosomes to lysosomes depends on the
vacuolar H+-ATPase (van Weert et al, 1995). Endosomal Carrier Vesicle (ECV) formation from endosomes is inhibited by bafilomycin treatment, whilst initial internalization and recycling are essentially unchanged. The mechanism by which internal pH alters these properties remain unclear. It seems that the requirement for an acidic endosomal lumen reflects the existence of an allosteric transmembrane pH-sensor governing the assembly of the budding apparatus at the cytosolic surface of the early endosomes. Consistent with this, is the finding that ε-COP associates with endosomes in a pH-sensitive manner and is required for an in vitro assay of ECV formation.

**Acidification of phagosomes**

Very similar to acidification of endosomes, maintenance of phagosomal pH is essential for their function. There are many studies which have focused on characterization of phagosomal pH utilizing various pathogen models such as Mycobacterium and Staphylococcus. Characterization of phagosomal pH using ingestion of FITC-labelled *Staphylococcus aureus* has been carried out in thioglycolate-elicited murine macrophages. After ingestion, the pH of the phagosomal compartment decreased rapidly, reaching a steady state value of 5.8-6.1 (Lukacs et al, 1990).

The vacuolar H+-ATPase plays very critical role in regulating the phagosomal pH as they are quintessential for the maintaining the pH like in endosomes. The substrate requirement and inhibitory profile of phagosomal acidification suggested that proton translocation across the phagosomal membrane was mediated by a vacuolar H+-ATPase (Lukacs et al, 1990). Phagosomal acidification is detectable
before phagolysosomal fusion occurs and prior to the appearance of lysosomal enzyme activity in the phagosomes (Lukacs et al, 1990; McNeil et al, 1983). It seems, however, that the vacuolar H+-ATPase accumulates in the phagosomal membrane as it matures within the cell (Caron and Hall, 2001).

Whereas the vacuolar H+-ATPase appears to be the main determinant of phagosomal acidification, there are other proton pumps present in the phagosomes which could also play essential role in this process. Fractionation studies have revealed the incorporation of Na+/H+ exchanger 1 (NHE1) into the phagosomal membranes. NHE1 is the predominant isoform of the NHE in macrophages, which are ubiquitous membrane transport proteins that play a major role in the regulation of intracellular pH and of cellular volume. NHE catalyzes the exchange of extracellular Na+ for intracellular H+, a process. Measurements of pH indicate that NHE1 is functional in the phagosomal membrane. NHE could therefore contribute importantly to phagosomal acidification. However, it has been shown that it is not the case, and this has been supported by the finding that the phagosomes are virtually devoid of Na+/K+ ATPases. Considering the latter observation, it is conceivable that mature phagosomes attain a much more acidic pH than endosomes due, at least in part, to the effective removal of Na+/K+ ATPases (Hackam et al, 1997). In conclusion, NHE, but not Na+/K+ ATPases, are present in phagosomes. Although present in the phagosomal membrane in a functional state, NHE1 does not contribute to the acidification of phagosomes, which is made by vacuolar H+ -ATPases (Hackam et al, 1997).
Acidification of the phagosomal compartment is crucial to its microbicidal functions. There are various mechanisms by which reduced pH of phagosomes can contribute to pathogen degradation and removal. The elevated proton concentration is directly lethal for certain microorganisms. It also helps in blocking pathogen survival by not allowing them to proliferate. Secondly, acidification promotes the spontaneous dismutation of superoxide to hydrogen peroxide which also acts as microbicidal. Also this mechanism is proving to be crucial in eliminating the toxic free radicals from the cellular environment. Furthermore, maintaining the reduced pH is a prerequisite for phagosome-lysosome fusion and it provides optimal conditions for the activity of certain hydrolytic enzymes (Lukacs et al, 1990).

**Acidification of Autophagosome**

Process of autophagy in eukaryotes is very complex and, debatably, can be divided in to the three parts based on autophagosome structure and function. Recently formed double membrane structure is described as autophagosome which does not have functional degradative capacity. In the subsequent series of events which are collectively termed as autophagosome maturation, autophagosomal pH reduces and also autophagosome acquire degradative capacity (Fig.7) In the final step autophagosome fuses with either endosomal compartment or lysosomal compartment delivering the degradative cargo material enwrapped in the autophagosome (Dunn, 1990).

Above described series of events are not well understood and there are not many reports suggesting that autophagosome goes through acidification and maturation process before fusing with either late endosome or lysosome (Eskelinen, 2005).
very few reports suggests that just formed autophagosomes are less acidic and hence are immature. As autophagosome gets mature, there is progressive decrease in pH and autophagosome gets the capacity to form amphisome or autolysosome. These fused vesicular structures have capacity to degrade engulfed cytoplasmic cargo material. Historically, autophagosome biogenesis has been topic of extensive investigation but studies on later stages of autophagy including putative autophagosome maturation process has not received sufficient attention. Only recently, with the advent of molecular characterization of autophagy genes and molecular dissection of various stages of autophagy, we have started giving due consideration to autophagosome maturation and autophagosome-lysosome fusion (Chen et al., Eskelinen, 2005).
Fig. 7 Autophagosome Maturation: Process of Autophagy is initiated by the nucleation of an isolation membrane or phagophore. Isolation membrane then elongates and closes on itself to form an double membrane vesicle called autophagosome. In most cases, once the autophagosome has been formed it receives input from the endocytic pathway (early and late endosomes and multivesicular bodies-MVB). These steps are collectively termed maturation. The amphisomes that result from the fusion of autophagosomes with late endosomes/MVB are acidic and hydrolytic vacuoles.
Chapter III

Materials and Methods
Reagents and Antibodies

All trans-retinoic acid (ATRA) was obtained from Sigma (no. R2625). CD2665, a selective RARβ/γ/α antagonist (no. 3800) and docosahexaenoic acid (DCHA), a selective retinoid X receptor (RXR) agonist (no. 3687) were purchased from Tocris Biosciences, Bristol, UK. Rapamycin (no. 553210) was procured from Calbiochem (La Jolla, CA). Retinoids were dissolved in DMSO at a concentration of 10mM and were stored under N2 in the dark at –80°C. Stock solutions were diluted to the appropriate concentrations with growth medium just before use. The antibody against light chain 3 protein (LC3) (nno. M115-3; clone no. 51-11) was from MBL International Corp.; the monoclonal antibodies against CIMPR for use in immunofluorescence staining were from Abcam (no. 2733-100, clone 2G-11), and for Western blotting, from Biolegend (no. 315902, clone-MEM238); the rabbit polyclonal antibody against Atg6/Beclin-1 (BECN1) was from Abgent (no. AP-1818b). The rabbit anti-mTOR polyclonal antibody was from Sigma (no. T2959). The rabbit monoclonal anti-phospho-mTOR (no. 2971), rabbit monoclonal antibody against Akt (pan) (no. 4691; clone-C67E7), and anti-phospho-Akt1 (no. 4058; clone-193H12) were from Cell Signaling Technologies. Anti-GFP rabbit polyclonal antibody (no. sc-8334), anti-actin mouse monoclonal antibody (no. sc-8432), goat anti-mouse-IgG-HRP (no. sc-8432), and bovine anti-rabbit-IgG-HRP (no. sc-2370) were from Santa Cruz Biotechnology. For immunocytochemistry, Alexa Fluor-555 goat anti-mouse IgG (no. A21422) secondary antibody was from Molecular Probes, Eugene, Oregon. LysoSensor Green DND-189 (no.L-7535), a pH indicator probe, was from Molecular Probes.
Cell Lines

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, 10mM sodium pyruvate, 2mM glutamine, 50 IU/ml penicillin, and 50µg/ml streptomycin, in a humidified 95% air/5% CO₂ atmosphere at 37°C. For generation of the CFP-LC3 stable cell line, HeLa cells were transfected by a plasmid encoding CFP-LC3.

The transfected cells were initially selected with G418 (Sigma; cat# G8168) at 500µg/ml of culture media for 2 weeks. Fluorescent cells were subsequently sorted with FACS, and individual clones were isolated by the limited-dilution method. Stable clones were maintained in medium supplemented with 300µg/ml of G418. In all experiments ATRA was used at 1 µM. All transfections were performed by using TransPass HeLa transfection reagent (no.M2556S) from New England Biolabs (NEB).

Fluorescent Expression Plasmid Vectors

Plasmid encoding CFP-LC3 used in this study was previously generated in our laboratory and has been described (Marvakis et al., 2006). mCherry-LC3 was prepared by digesting pCFP-LC3 with EcoR1/BamH1 and subcloning of the LC3 ORF into the EcoR1/BamH1 sites of pmCherry-C1 (Clontech). The pmCherry-GFP-LC3 construct was a generous gift from Dr. Terje Johansen. Lamp1-mRFP plasmid construct was obtained from Addgene (Addgene plasmid 1817). pCerulean-Rab5,
pCerulean-Rab7, and pCerulean-Rab9 constructs used in this study were generated in our laboratory and described previously (Marvakis et al., 2006).

**Cloning of CI-MPR**

For generating CI-MPR mGFP and mRFP fusion constructs, cellular mRNA was isolated from HeLa cells by using the Trizol method. CI-MPR cDNA was prepared by using Protoscript-II RT-PCR kit (no.E6400S) from NEB. Amino acids 110-2491 from the CI-MPR ORF were directly amplified from the cDNA by using a forward primer (ctggaattcaacacaacagtg) containing the only *Eco*RI site in the sequence and a reverse primer (ctgaccggtaaGATgtgtaaggtcctcgct) which contains a unique *Age*I site. The three capital letters indicate in reverse orientation the last amino acid of the CI-MPR ORF. The PCR product was digested with *Eco*RI/*Age*I and cloned in frame at the *Eco*RI/*Age*I sites of plasmid mGFP-N1. The ORF encoding the first 109 amino acids and also a Kozac sequence and *Xho*I/*Eco*RI flanking sites were artificially synthesized (Genescript Inc.) and subcloned in the *Xho*I/*Eco*RI sites of the mGFP-N1 vector containing amino acids 110-2491, thus creating CI-MPR-mGFP. An *Xho*I/*Age*I site from the last vector was also subcloned in the *Xho*I/*Age*I sites of plasmid mRFP-N1 to create CI-MPR-mRFP. These constructs therefore express the full ORF of CI-MPR, including the signal peptide, and the fluorescent tag is placed in the c-terminus of CI-MPR. The c-terminus of CI-MPR is exposed toward the cytosol, and fluorescent tags such as GFP are not subject to pH-mediated quenching in acidified organelles.
siRNA/ RNA interference Oligomer Sequence

HeLa cells were transfected with annealed double-stranded Silencer select validated siRNA against CI-MPR from applied biosystem (siRNA ID no. s7217) as designated in the experiments by using the manufacturer's suggested protocol. The sequence for the siRNA used is CUACCUGUAUGAGAUCCAAtt (sense) and UUGGAUCUCAUACAGGUAGtt (antisense). Chemical treatments in transfected cells were initiated 8 h after transfection, and cells were analyzed 48 h after transfection.

General Methods

To obtain total cell lysates after a designated incubation period with various treatments, cells were washed twice with ice-cold PBS, lysed in RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate (SDS), protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktail], and clarified by high-speed centrifugation. For Western blot analysis, equal amounts of total protein were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with blocking buffer (TBST-1% casein) for 1 h, probed with primary antibodies for 2 h and then incubated with the HRP-conjugated secondary antibody for 1 h. Antibody binding was detected by enhanced chemiluminescence (Amersham). Density for each band was analyzed by using a densitometer. Equal protein loading was confirmed by probing against β-actin. Values obtained for phosphorylated mTOR and phosphorylated Akt1 were normalized to total mTOR and total Akt density.
respectively. For immunofluorescence, cells were grown on chambered coverslips. At the end of the experimental treatment, the cells were fixed with 4% paraformaldehyde in 0.1 $M$ phosphate buffer (15 min), washed with wash buffer (PBS/0.1% saponin), blocked for 1 h with blocking buffer (PBS/1% BSA/0.1% saponin), and sequentially incubated with the primary and secondary antibodies in blocking buffer. After the final wash, the chambers were filled with mounting media (80% glycerol, 100 mM Tris, pH 8.0) containing antifade (DABCO) and analyzed by confocal microscopy.

**Confocal Microscopy**

For colocalization studies, cells were seeded overnight in Lab-Tek chambers (Nalgene Nunc, Rochester, NY) and co-transfected with the plasmids of interest or siRNA targeting CIMPR gene, by using TransPass HeLa transfection reagent. Confocal microscope images of cells 24 to 48 h after transfection were captured on a Zeiss LSM 510 confocal microscope or by using the 458-nm line of an Ar laser with a 465–505 emission filter for CFP, the 488-nm line of an Ar laser with a 505–550 emission filter (GFP), a 543-nm HeNe laser line with a 560–615 emission filter line for mCherry/mRFP. Images were captured with a Plan-Achromat 1.4 NA 63× oil-immersion objective. Cells expressing both proteins were selected for z-sectioning. Z stacks were taken by using a pinhole of 0.5 Airy units for both channels. Images were analyzed with ImageJ and Zeiss Image Examiner software and prepared by Adobe Photoshop 7.0.
Statistics

Results are presented as the mean ± SEM from three independent experiments. Two group comparisons were performed by using Student's $t$ test. Multiple group comparisons were performed by using one-way analysis of variance and Fisher's least significant difference.
Chapter IV

Consequences of Treatment of Retinoic acid to HeLa Cells
Introduction

Nutrient availability is one of the best-characterized factors involved in regulation of autophagy. The great sensitivity of the autophagic response to the nutritional state implies that certain macro- or micronutrients regulate this response. In general, macronutrient (proteins, carbohydrates, lipids) availability suppresses autophagy. With the exception of certain vitamins, the effect of various dietary micronutrients on autophagy has not been examined. Most vitamins studied have been found to stimulate autophagy. Vitamin A metabolite, Retinoic acid (ATRA), has diverse biologic effects in the control of cell growth and differentiation. Despite our knowledge of the diverse effects of retinoic acid on several cellular functions, its effect on autophagy has not been studied. In our present research, we proposed to study the possible functional relationship of vitamin A with autophagy pathway. In order to investigate the putative role of ATRA on autophagy, we analyzed the effects of ATRA treatment on the expression of autophagosome marker LC3 fused with various fluorescent marker genes (CFP, mCherry) in dividing HeLa cells. Autophagy is very quick process and autophagosome has a half life of 10 min. To investigate the autophagosome spatially and temporally, we generated HeLa stable cell line expressing CFP-LC3. Autophagosome biogenesis was studied by following the LC3 expression pattern after ATRA treatment. Autophagosome maturation process was studied by LC3 sub cellular localization by confocal microscopy following treatment of ATRA.
**ATRA Reduces the Number of Non-acidified Autophagosomes**

To investigate the role of retinoic acid and analogues in autophagy, we initially generated a stable HeLa cell line expressing LC3 fused to the carboxy terminus of CFP (LC3-CFP). Attempts to generate LC3- EYFP, LC3- GFP, or LC3-mCherry stable cell lines were unsuccessful, presumably because of the toxic effects of these fluorescent proteins when expressed over the long term as LC3 fusion proteins. The stable cell line is expressing CFP-LC3-I, which exhibits diffused cytosolic and nuclear distribution. Cleavage of a small carboxy-terminus portion from LC3-I and lipid conjugation generates LC3-II, a protein that has been shown specifically to associate with double membranes of maturing autophagosomes (AUTs) and decorates both the cytosolic and luminal sides of the organelle (Bampton et al., 2005; Eskelinen, 2005; Kabeya et al.,2000; Marvakis et al., 2006), thus appearing as distinct punctuate structures. The ratio of LC3-I to LC3-II correlates very well with the total number of AUTs present at any time, and for that reason has been extensively used as measurement for steady-state levels of maturing AUTs. The ratio of LC3-I/LC3-II is not informative on the maturation level of AUTs or the degree of their acidification.

Treatment of HeLa-LC3 cells with 1 µM ATRA for 48 h resulted in substantial reduction of CFP-LC3-positive structures, whereas treatment with 0.5 µM of rapamycin (a known activator of autophagy) induced a robust increase of CFP-LC3 structures (**Fig. 8**).
FIG. 8 Effect of ATRA and Rapamycin on abundance of AUTs in Stable HeLa Cells: CFP-LC3 stably transfected HeLa cells (CFP-LC3-S) were treated with ATRA (1.0 µM) or Rapamycin (0.5 µM) for 48 hrs. The experiment was repeated three times.

To confirm that this observation is not limited to the stable cell line, we treated CFP-LC3 transiently transfected HeLa cells and obtain similar results (Fig. 9).

Fig. 9 Effect of ATRA and Rapamycin on abundance of AUTs in transiently transfected HeLa Cells: CFP-LC3 transiently transfected HeLa cells (CFP-LC3-T) were treated with ATRA (1µM) or Rapamycin (0.5 µM) for 48 hrs.
**Fig. 10** Quantification of the number of AUTs per HeLa cell: CFP-LC3-S (stable cell line), mCherry-LC3-T (transiently transfected) and CFP-LC3-T (transiently transfected) cells treated with ATRA or Rapamycin (Rapa) or CNT (Left untreated). For quantification, cells were optically sliced on a confocal microscope and then maximal-intensity z-projections were generated for each cell to visualize all AUTs. LSM images were exported as TIFF files, and the number of AUTs was quantified by using Image J. The data are presented as the average number of AUTs in each group. Treatments with different superscripts are statistically different (b>a, b>a1, a>a1>a2, b1>a1). Data shown are the mean ± SEM from three independent experiments.

In both cases, CFP-LC3 stably and transiently transfected HeLa cells, ATRA reduced CFP-LC3 structures by almost 50% ([Fig. 10](#)) but did not affect the ratio of CFP-LC3-I to CFP-LC3-II ([Fig. 11](#)). Rapamycin treatment though resulted in a substantial reduction of the CFP-LC3-I to CFP-LC3-II ratio.

Surprisingly, ATRA treatment did not affect the abundance of mCherry-LC3 positive AUTs in transiently transfected HeLa cells ([Fig. 12](#)). Given that CFP is very susceptible to quenching under acidic conditions, whereas mCherry is not, our data
suggested that ATRA either increases acidification of autophagosomes (amphisomes) or increase the autophagosome–lysosome fusion rate.

**Fig. 11 Western Blott analysis of whole cell lysate collected from CFP-LC3-S HeLa cells:** Parallel cultures of CFP-LC3-S cells were collected after 48 hrs of treatment, and total cell lysates were separated by SDS-PAGE, and subjected to immunoblotting with anti-LC3 and anti-β-Actin. The results are representative of three independent experiments. The intensity of the bands was quantified by Image J, and the ratio of LC3-I to LC3-II was calculated. Bars represent the mean ± SEM from three independent experiments. The intensity of the bands was quantified by Image J, and the ratio of LC3-I to LC3-II was calculated. Bars represent the mean ± SEM from three independent experiments.
HeLa cells: mCherry-LC3 transiently transfected HeLa cells were treated with ATRA (1 µM) or Rapa (0.5 µM) for 48 h.

**ATRA does not affect the levels of upstream regulators of autophagy**

Induction of AUT biogenesis requires two complexes. The first one, that initiates vesicle formation, contains the class III PI3K (Vps34), Beclin-1/Atg6, Atg14, and Vps15/p150 and is highly controlled by Beclin-1. Beclin-1, the mammalian homologue of ATG6, was the first protein shown to be indispensable for autophagy in mammals (Bonifacino and Rojas, 2006; Kihara et al., 2001; Liang et al., 1999). The second complex, responsible for the vesicle nucleation, contains Atg1/ULK1, mAtg13, mAtg13-associated proteins FIP200, and Atg101 in mammalian cells, the association of which is controlled by mTOR (Mizhushima, 2010). Autophagy is negatively regulated by the serine/threonine kinase mTOR (mammalian target of rapamycin) (Marvakis et al., 2008; Wullschleger et al., 2006). Phosphorylated mTOR (pmTOR) is part of the induction complex and acts as a negative regulator of
autophagy (Schmelzle and Hall, 2000). In addition, activated mTOR induces hyperphosphorylation of Atg13, which reduces its binding affinity to other Atg interacting proteins, thereby inhibiting autophagy (Kamada et al., 2000). One well-characterized pathway for mTOR activation involves Akt1 activation. Akt1 phosphorylates and inhibits the tuberous sclerosis complex 2 (TSC2) (Potter et al., 2002). TSC2 negatively regulates mTOR by acting as a GTPase-activating protein (GAP) for the small GTPase Rheb, which binds and activates mTOR (Long et al., 2005). Therefore, elevated levels of phosphorylated Akt1 (pAkt1) promote conditions that inhibit autophagy.

To investigate whether ATRA affects the levels of AUTs by inhibiting induction of autophagosome formation, we examined the levels of Beclin-1, pmTOR, and pAkt1 in HeLa cells treated for 12, 24, and 48 h. Our results indicate that ATRA did not affect the total levels of Beclin-1, Akt1, Akt2, Akt3, and mTOR, and neither the levels of pmTOR and pAkt1 (Fig. 13). These data suggest that ATRA does not inhibit induction of AUT formation.
Fig. 13 Protein Expression Analysis of critical regulators of autophagy by Western Blot: HeLa cells were treated with ATRA (1mM) for the indicated time points. At the end of the incubation period, total cell lysates were prepared, and equal amounts of proteins were separated on SDS-PAGE. Levels of Beclin-1, mTOR, phospho-mTOR, pan (Total)Akt, and phospho-Akt1 were evaluated with immunoblotting. Phosphorylated-mTOR levels were assessed by using a phospho-mTOR antibody specific for phosphorylation on Ser2448, and phosphorylated-Akt1 levels were assessed by using a phospho-Akt1 antibody specific for phosphorylation on Ser473. β-actin was used as a protein-loading control.
Discussion

Turnover of most long-lived proteins, macromolecules, biologic membranes, and whole organelles, including mitochondria, ribosomes, endoplasmic reticulum, and peroxisomes, is mediated by autophagy. A basal form of autophagy is essential for maintenance of cellular homeostasis, and an inducible form becomes activated under stress conditions (nutrient deprivation, infections, and toxins). The overall process of autophagy can be generally divided into three parts; induction of the autophagosomal membranes, functional and structural autophagosome maturation, and fusion with lysosomes. Starvation and nutrient regulation of autophagy is well documented. There have been many reports of studying the effects of macromolecules on autophagy. There are very few reports which describe autophagy in context of micronutrients regulation. In the efforts to study the effect of Vitamin A on autophagy, our initial experiments suggested that retinoic acids are not involved in autophagosome biogenesis, but may play a crucial role in autophagosome acidification and maturation. Treatment of ATRA to stable transfected and transiently transfected HeLa cells showed that CFP-LC3 positive structures were reduced. Contradictory to the previous confocal experiments, we were not able to see any difference in the expression level of LC-II at various time points in the HeLa cells. As it is well documented that CFP loses its fluorescence in the more acidic environment (late autophagosome or amphisomes or autolysosomes), we speculated that we were not able to observe the complete set of profile of autophagosomes (non acidified and acidified) in the CFP-LC3 HeLa cells in the confocal experiments. To observe the complete set of autophagosome under confocal microscope, in next experiment we
change the fluorophore to mCherry. mCherry has pKa value < 4.5 and hence tolerates acidic environment very well. In the next experiment ATRA treatment to mCherry-LC3 HeLa cells, we did not observe the reduction in the number of autophagosome what we had earlier observed in the CFP-LC3 HeLa cells. This last piece of data was consistent with the expression profile of LC3-II observed in the western blot experiments. Further, treatment of ATRA to HeLa cells does not change the expression level of key critical upstream modulator of autophagosome biogenesis including mTOR, AKT and beclin-1. These initial data indicated that ATRA might be involved in the later of autophagy i.e. autophagosome maturation and autophagosome-lysosome fusion. In the quest to look in to the later maturation event during the process of autophagy, in the next experiment we transfected the HeLa cells with pH sensitive reporter plasmid mCherry-GFP-LC3. Observation under confocal microscope revealed reduction in the number of yellow dots( immature non acidified autophagosome) and more number of the red dotted ( mature acidified autophagosome) structures. pH sensitive reporter plasmid transfection experiments provided definitive evidence of involvement of ATRA on autophagosome maturation. The mechanism of autophagosome maturation by ATRA and receptor involved in the process is described in the next chapter.
Chapter V

Mechanism of Autophagosome Maturation by ATRA
Introduction

The ATRA has diverse physiological functions in the various cell types. ATRA carried out these functions by regulating the expression of genes through two families of nuclear receptors, the RA receptors (RARs) and the retinoid X receptors (RXRs). There are many functions of ATRA which cannot be attributed to above mentioned nuclear receptor. Retinoids are also frequently used pharmaceuticals. Key drawbacks for using these compounds are there non-specific functions and undesirable side effects. In addition to the effects of retinoic acid through RARs and RXRs, there are evidences which suggest that other retinoid response pathways that are independent of the nuclear receptors may exist (Kang et al., 1997; Kang et al., 1998). Interestingly, previous studies have shown direct binding of RA to the cation-independent mannose-6-phosphate/IGFII receptor (CIMPR) (Kang et al., 1997).

In our initial studies data suggested that ATRA treatment to HeLa cells do not affect autophagosome biogenesis. Instead, treatment of HeLa cells reduces the number of non-acidified or immature autophagosomes. These observations lead us to hypothesize that ATRA treatment might induce the autophagosome maturation or later stages of autophagy. To further understand the underlying mechanism of autophagosome maturation by ATRA we studied involvement of nuclear receptor (RAR and RXR) by using receptor specific agonist and antagonist. We analysed the expression and subcellular localization of LC3 in presence of specific agonist to RXR receptor and specific antagonist to RAR receptor after treatment of HeLa cells with ATRA. Involvement of CI-MPR in autophagosome maturation was studied by co-
transfecting GFP-CI-MPR and mRFP-CI-MPR in HeLa cells and by employing siRNA against CI-MPR.

**Role of Nuclear Receptor in AUT maturation By ATRA:**

To investigate whether the effect of ATRA on AUT maturation is mediated by the classic retinoid acid receptors, we perform studies in the presence of receptor-specific antagonists and agonists. Two distinct classes of nuclear retinoid receptors are termed RARs and RXRs, each of which has three distinct subtypes, α, β, and γ. The RARs bind ATRA and 9-cis-RA with high affinity, whereas the RXRs bind 9-cis-RA selectively (Chambon, 1996). At high doses, though (>10 µM), ATRA can slightly transactivate RXRs.

Treatment of CFP-LC3 stably transfected HeLa cells with CD2665 (10 µM) did not block the effect of ATRA (1 µM) in reducing the number of CFP-LC3 structure (Fig. 14), and treatment with CD2665 alone had no effect. CD2665 is a selective RARα/β/γ antagonist, with KD values of 0.1, 0.3, and 1 µM for RARγ, RARβ, and RARα, respectively (Kim et al., 2000). Actually, the effect of ATRA in reducing the number of CFP-LC3-positive structures was even more pronounced in the presence of CD2665. Binding of CD2665 to RARs could increase the bioavailability and binding of ATRA to another target. Although the effect of ATRA is not expected to be mediated by the RXRs, we subjected CFP-LC3 HeLa cells to treatment with DCHA, a highly potent and specific agonist of RXRs, and did not observe any significant
effect. These results strongly suggest that the effect of ATRA is not mediated by the classic RARs and RXRs receptors.

![Cell images](images)

**Fig. 14 Role of Nuclear Receptors (RAR and RXR) in AUT maturation by ATRA:** The effect of ATRA on AUT maturation is not mediated by the RARα/β/γ and RXR receptors. CFP-LC3 stably transfected cells were cultured on chamber slides.
and treated with 1 mM ATRA, 10 mM CD2665, 10 mM DCHA, or 1 mM ATRA plus 10 mM CD2665 in combination. The number of AUTs per cell in each group was measured as described earlier. Data shown are the mean ± SEM from three independent experiments.

**mCherry-GFP-LC3 pH-sensitive reporter Assay**

CFP-, EYFP- and GFP-LC3 are being widely used as markers of maturing AUTs. However, these fluorescent proteins are acid labile with a $pK_a$ of 6.0 (Shaner et al., 2005), making it impossible to monitor AUTs after they become acidified (amphisomes) or fused with lysosomes. The luminal pH of late endosomes have a pH of about 5.5, and lysosomes have a pH of about 4.7 (Zen et al., 1992). Fusion of the monomeric red fluorescent protein mCherry to LC3 alleviates some of these problems. The $pK_a$ of mCherry is $< 4.5$, making the protein very acid stable. As we showed earlier in this study, the number of mCherry-LC3–positive AUTs is much higher than that of CFP-LC3–positive AUTs. Simultaneously to detect both nonacidified and acidified AUTs, a mCherry-GFP double-tagged LC3 strategy recently was devised (Pankiv et al., 2007). Expression of mCherry-GFP-LC3 results in yellow-punctuated structures, which are nonacidified AUTs, and red structures, which are either amphisomes or autophagolysosomes. In the present study, treatment of mCherry-GFP-LC3–transfected HeLa cells resulted in substantial shift of the number of yellow-punctuated AUTs in favor of red-labeled AUTs (Fig. 15). After 48 h of ATRA treatment, almost half of the AUTs displayed red fluorescence only, thus confirming our earlier hypothesis that ATRA promotes acidification of maturing AUTs or increases the rate of autophagosome-lysosome fusion.
**Fig.15 pH-sensitive reporter Assay:** ATRA treatment induces acidification of AUTs, as evaluated by using the pH-sensitive reporter GFP-mCherry-LC3. HeLa cells were transfected with GFP-LC3-mCherry, and either left untreated or treated with 1 mM ATRA for 24 and 48 h, respectively. After the indicated period of
treatments, cells were prepared for live imaging by using confocal microscopy. The number of yellow and red punctuate structures was determined as described earlier. The data are presented as percentage of yellow puncta in control cells or cells treated with ATRA for the indicated time points. Data shown are the mean±SEM from three independent experiments.

**Role of CI-MPR in AUT Acidification/ Maturation by ATRA**

As we showed, the effect of ATRA in promoting acidification of AUTs is not mediated by the classic RAR and RXR receptors. Earlier studies identified CI-MPR as a target for retinoic acid, and showed that retinoic acid can induce translocation of the receptor from perinuclear compartments to uncharacterized vesicular structures. The best-described function of CI-MPR is to transport mannose-6-phosphate (M6P)-bearing glycoproteins from TGN to endosomal/prelysosomal compartments. The CI-MPR is one of two transmembrane proteins that bind M6P tags on acid hydrolase precursors in the TGN that are destined for transport to the lysosome. The other protein is the cation-dependent mannose-6-phosphate receptor (CD-MPR). Newly synthesized lysosomal enzymes are posttranslationally modified to contain M6P residues on their N-linked oligosaccharides. The M6P residues enable enzymes to bind to CIMPR (and CD-MPR) receptors in the TGN. The ligands bind in one of the repeats of the large luminal domain. The receptor/ligand complexes cluster into clathrin-coated transport vesicles and travel to acidic prelysosomal compartments where the low pH causes dissociation of the receptor–ligand complex. The free M6P receptors can travel to the plasma membrane or back to the TGN to reinitiate another cycle of biosynthetic enzyme transport.
To determine whether RA induces translocation of the CI-MPR in maturing autophagosomes, we prepared mGFP- and mRFP-tagged CI-MPR constructs. The fluorescent tag was inserted in the carboxy terminus of CI-MPR, which is the cytoplasmic domain of the protein, to ensure that acidified organelles will not quench the fluorescent signal. We initially transfected the CFP-LC3 stable HeLa cell line with CI-MPR-mRFP and examined whether it colocalizes with AUTs in ATRA-treated (Fig. 16b) or -untreated cells (Fig. 16a). CI-MPR-mRFP was localized mostly in perinuclear compartments (presumably TGN), some vesicular compartments, and plasma membrane. Treatment with ATRA induced a significant redistribution of CI-MPR-mRFP to peripheral vesicular compartments, but those were not CFP-LC3–labeled AUTs (Fig. 16b).
Fig. 16 Colocalization study of CFP-LC3 and CIMPR-mRFP in HeLa Cells: CFP-LC3 stably transfected HeLa cells, treated with ATRA (16b) or untreated (16a), were transfected with CIMPR-mRFP; 48 h after transfection, live cell imaging was performed with confocal microscopy.

We also co-transfected ATRA treated and untreated HeLa cells with mCherry-LC3 and CI-MPR-GFP. Even in the absence of ATRA, we noticed some colocalization of CI-MPR-GFP with mCherry-LC3–labeled AUTs (Fig. 17a). In ATRA-treated cells, significant translocation of CI-MPR-GFP in mCherry-LC3–labeled AUTs was noticed (Fig. 17b). These data show that retinoic acid induces redistribution of CI-MPR in acidified AUTs or autophagolysosomes.

Fig. 17 Colocalization study of mCherry-LC3 and CIMPR-mGFP in HeLa cells: ATRA treatment induces redistribution of CIMPR from TGN to acidified LC3-positive structures: HeLa cells, either treated with ATRA (17b) or left untreated
(17a), were co-transfected with mCherry-LC3 and CIMPR-mGFP and subjected to confocal microscopy. The images are Z-sections and obtained as described earlier.

In the previous experiments, we showed that ATRA induces the translocation of CIMPR in an acidic compartment that is also positive for LC3. This compartment can be either an amphisome or an autophagolysosome. Autophagolysosomes are also positive for LAMP-1, a lysosomal marker protein. Transfection of ATRA-treated HeLa cells with CIMPR-GFP and LAMP-1-RFP and confocal microscopy indicated that CIMPR is not recruited in LAMP-1 positive structures (Fig. 18). This result suggests that retinoic acid induces redistribution of CIMPR in amphisomes (acidified AUTs) and not in autophagolysosomes.
Fig. 18 Colocalization study of CIMPR-mGFP and LAMP1-mRFP in HeLa cells: HeLa cells were co-transfected with CIMPR-mGFP and LAMP1-mRFP and either treated with 1 mM ATRA or left untreated. At 48 h later, cells were analyzed by using confocal microscopy and Z-sectioning. This images shows that ATRA does not redistribute CIMPR to LAMP1-positive organelles.
Role of endogenous CI-MPR in AUT Acidification

Our studies suggest that CI-MPR may play a critical role in mediating the effects of retinoic acid on AUT maturation. We next asked whether downregulation of the endogenous levels of CI-MPR could affect autophagy. We used a company-validated siRNA against CI-MPR in the CFP-LC3 stable cell line and were able to document a dose-dependent downregulation of endogenous CI-MPR protein levels (Fig. 19).

**nM concentration of siRNA**

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*Fig. 19 Western Blot analysis of expression of CI-MPR in HeLa Cells After siRNA-mediated knockdown of CI-MPR:* HeLa cells were cultured on six-well plates and transfected with increasing concentrations (0, 2.5, 5.0, and 10 nM) siRNA against CI-MPR. Cells were lysed 48 h after transfection, and equal amounts of whole-cell lysates were separated with SDS-PAGE. CI-MPR levels were measured with immunoblotting by using a monoclonal anti-CIMPR antibody (clone MEM-238). β-actin immunostaining served as a proteinloading control.
Transfection of CFP-LC3 cells with 10 nM (final concentration) siRNA resulted in a remarkable decrease in the ratio of CFP-LC3-I to CFP-LC3-II (Fig. 20).

**Fig. 20 Western blot analysis of LC3 expression after CI-MPR knockdown in HeLa cells:** CFP-LC3 stably transfected, grown on six-well plates, were transfected either with 10 nM siRNA against CI-MPR or mock transfected by using a scrambled siRNA and treated with 1 mM ATRA or left untreated. 48 h after transfection total cell lysates were obtained and subjected to Western blot analysis by using an anti-LC3 antibody.

Visualization of the CI-MPR knockdown HeLa cells under confocal microscope also showed a large accumulation of LC3-CFP–positive structures. The siRNA-CI-MPR mediated increase of AUTs could not be reversed by ATRA, further suggesting that the effect of retinoic acids on AUTs is mediated primarily by CI-MPR. Immunocytochemistry for endogenous CI-MPR in siRNA-transfected CFP-LC3 cells indicated that complete silencing was observed in more than 80% of the
cells. Remarkably, the few cells found in which silencing of CI-MPR was not effective had normal levels of AUTs (Fig. 21).

**Endogenous CI-MPR**

**LC3-CFP**

Fig. 21 siRNA-mediated knockdown of CI-MPR in HeLa cells: CFP-LC3 cells grown on chambered coverslips were transfected with 10 nM siRNA against CI-MPR. At 48 h after transfection; the cells were fixed and processed for immunofluorescence staining by using an anti-CI-MPR monoclonal antibody (clone 2G-11) and Alexa Fluor-555 goat anti-mouse fluorescent secondary antibody, and examined with confocal microscopy.

Transfection of HeLa cells with siRNA against CI-MPR and the pH-sensitive reporter mCherry-GFP-LC3 indicated that accumulated AUTs were nonacidified AUTs (yellow punctuates) (Fig. 22).
Fig. 22 CI-MPR knockdown in HeLa cells transfected with mCherry-GFP-LC3 pH-sensitive reporter plasmid: HeLa cells grown on chambers were treated with ATRA or left untreated and co-transfected with GFP-mCherry-LC3 and siRNA against CI-MPR.

Treatment with ATRA resulted in a slightly decreased ratio of yellow-to-red punctuates (Fig. 23), presumably because of incomplete CI-MPR silencing in a portion of cells. This last piece of data suggests that CI-MPR may be involved in AUT acidification, and that maturation is a prerequisite for physiologic turnover of newly synthesized AUTs.
Fig. 23 Quantification of Autophagosome in CI-MPR knockdown HeLa cells: The number of yellow and red punctate structures was estimated by using confocal microscopy, as described previously. The total number of AUTs and the percentage of yellow puncta in each treatment group were analyzed separately. Treatments with different superscripts are statistically different. Data shown are the mean ± SEM from three independent experiments.

Effect of siRNA-mediated knockdown of endogenous CI-MPR on Endosomes and Lysosomes

To determine whether downregulation of CIMPR affects the abundance of endosomes or lysosomes or both, we co-transfected HeLa cells with siRNA against CIMPR and one of either cerulean-Rab5, cerulean-Rab7, cerulean-Rab9, or Lamp1-RFP. The cells transfected with Lamp1 were also stained with LysoSensor Green. We did not observe any effect on abundance of Rab5- and Rab7-positive endosomes. However, knockdown of CIMPR resulted in increased numbers of Rab9-positive endosomes (Fig. 24).
**Fig. 24** Effect of siRNA-mediated knockdown of CI-MPR on Rab9-positive endosomes in HeLa cells: HeLa cells grown on chambers were co-transfected with Rab9-Cerulean and siRNA against CI-MPR or a scrambled siRNA. At 48 h after transfection, the number of Rab9-positive structures was estimated by using the method described earlier.
Knockdown of CI-MPR also did not affect lysosome abundance, but reduced the number of Lamp1-positive structures that were also labeled with LysoSensor Green, indicating reduced lysosomal acidification (Fig. 25). It should be noted that LysoSensor Green stains other acidified structures in addition to lysosomes, and that, in addition to CI-MPR, the cation-dependent mannose-phosphate receptor (CD-MPR) is also involved in acidification of late endosomes. The contribution of each receptor in different compartments is unknown.

Fig.25 Effect of siRNA-mediated knockdown of CI-MPR on Lysosomes in HeLa cells: HeLa cells were cotransfected with Lamp1-RFP and siRNA against CI-MPR or a scrambled siRNA. At 48 h after transfection, the cells were incubated for 1 h with 2 mM LysoSensor Green, washed several times with warm medium, and visualized with confocal microscopy.
Our data clearly indicate that CI-MPR is involved in acidification of AUTs and at least partially in the acidification of lysosomes. Accumulation of Rab9-positive structure in cells with reduced CI-MPR may suggest that AUTs fuse with a subset of Rab9 endosomes, and may be dependent on this process for their acidification. However, Rab9-positive endosomes fuse also with lysosomes, and we have not observed significant co-localization of AUTs and Rab9-positive endosomes (Marvakis et al., 2008).

It could be that accumulation of Rab9-positive endosomes is due to their reduced fusion with lysosomes. Lysosomes do not carry CI-MPR, and their acidification depends primarily on their fusion with late endosomes. However, we found that acidified AUTs carry their own CI-MPR, and direct acidification of AUTs without fusion with endosomes cannot be excluded.
Discussion

The initial set of experiments revealed that ATRA induces autophagosome maturation. Physiological functions of ATRA in the cells are attributed to nuclear receptors RAR and RXRs, specifically to RARs. To look into the possible involvement of nuclear receptor in the physiological outcome of ATRA treatment to HeLa cells in the previous experiments, we used specific antagonist to RXRs and agonist to RAR. In the series of experiments with antagonist and agonist treatment to HeLa cells, we did not observe any difference in the autophagosome biogenesis and maturation. After these experiments we conclude that nuclear receptors are not involved in the autophagosome maturation in the HeLa cells.

There are reports that document the presence of non nuclear receptors for the ATRA. One such receptor which may be responsible for the physiological outcome in our experiments is CI-MPR. To study the possible involvement of CI-MPR in the autophagosome maturation, we generated GFP and mRFP tagged CI-MPR fusion proteins. In our initial observation with colocalization studies using confocal microscopy revealed that CI-MPR is possibly involved in the autophagy process. Autophagosomes maturation process consists of multiple fusion events with endosomal compartments. These fusion events are thought to be responsible for the enrichment of autophagosomes with the vacuolar-type proton ATPase that mediates acidification of AUTs to amphisomes. Therefore, one possible explanation for increased AUT acidification by ATRA is that ATRA increases the fusion rate of autophagosomes with endosomes. Alternatively, CIMPR may mediate the
transportation of the proton ATPase to autophagosomes directly from the Golgi network. In our subsequent CI-MPR knock down experiments, Our data showing accumulation of nonacidified AUTs after silencing of CI-MPR strongly suggest the involvement of this receptor in AUT acidification.

Based on electron microscopy, subcellular fractionation studies, and immunofluorescence studies, localization of endogenous CI-MPR in a subset of autophagosomes was previously reported (Dunn, 1990; Jahreiss et al., 2008). In agreement with these studies, we found fluorescently tagged CI-MPR to acidified AUTs in increasing amounts under ATRA treatment, which suggest that CI-MPR also may be involved in delivering acid hydrolases to these structures, creating degradative autophagosomes. Knockdown of endogenous CI-MPR leads to substantial accumulation of nonacidified AUTs, which further suggests that acidification and possible acquisition of hydrolases may be an important event in the maturation of all AUTs and not only amphisomes, and it may be a requirement for fusion with lysosomes.
Chapter VI

General Discussion and Future Prospective
**General Discussion**

Autophagy is an intracellular catabolic process that responds with great sensitivity to nutrient availability, implying that certain macro- or micro-nutrients are involved. We found that retinoic acid promotes autophagosome maturation through a pathway independent from the classic nuclear retinoid receptors. Retinoic acid redistributes the cation-independent mannose-6-phosphate receptor from the trans-Golgi region to maturing autophagosomal structures inducing their acidification. Manipulation of the autophagic activity by retinoids could have enormous health implications, since they are essential dietary components and frequently used pharmaceuticals. Although a better understanding of the affects of ATRA on autophagy utilizing CI-MPR had been obtained, at the time we started these studies the specific consequences of ATRA treatment were still not known. The goal of this work has been, therefore, to better understand the physiological outcomes of the ATRA treatment on autophagy and the underlying mechanism.

Vitamin A is an essential dietary component that is involved in several physiological processes, such as reproduction, embryonic development, tissue remodeling, vision and immune function. Vitamin A deficiency has been extensively linked to increased oxidative stress, inflammation and neurodegeneration and historically is considered as a nutritionally acquired immunodeficiency disease. Natural and synthetic retinoids play a major role in regulating cellular growth and differentiation, and can induce apoptosis in a wide variety of malignant cells. The
mechanism however by which retinoids protect against viral and bacterial infections and suppress tumorigenesis is still unknown.

Regulation of autophagy by macronutrient (proteins, carbohydrates, lipids) availability is well recognized. In general, amino acid deprivation directly triggers the autophagic response, whereas carbohydrates and lipids affect autophagy indirectly through the insulin/glucagon signaling pathway. With the exception of a few reports on vitamin D3 and selenium, the effect of micronutrients and particularly vitamin A on autophagy has not been studied. It is noteworthy, however, to mention that either vitamin A or autophagic deficiency can potentially lead to similar pathophysiological conditions.

To investigate the role of retinoic acid in autophagy, we initially subjected transiently and stably CFP-LC3 transfected HeLa cells to low micromolar doses of ATRA (all-trans retinoic acid) and noticed a decrease in the steady state levels of CFP-LC3-labeled autophagosomes by confocal microscopy. However, we did not observe any changes in the ratio of CFP-LC3-I to CFP-LC3-II by western blotting. Both procedures, though, gave similar results when the cells were treated with rapamycin, which induces autophagosome biogenesis. To determine whether ATRA could affect the steady state levels of autophagosomes by inhibiting their formation, we examined the protein levels of Beclin 1, phosphorylated mTOR and phosphorylated Akt1 under retinoid stimulation. Our studies suggested that retinoids do not affect the early stages of autophagosome formation. However, it is well accepted in the field that, upon biogenesis, autophagosomes progress through a series of maturation events before fusion with lysosomes and formation of autolysosomes.
Therefore, changes in the pH of the autophagosomal lumen or fusion with the acidic lysosomes could induce fluorescent quenching of CFP/GFP/EYFP fusion proteins. Indeed, treatment of mCherry-LC3 transfected cells (mCherry is acid resistant) with ATRA did not result in significant change in the number of autophagosomes/autolysosomes. To further verify this observation, we subjected GFP-mCherry-LC3 (pH sensitive reporter) transfected cells to ATRA and observed a significant reduction in the ratio of yellow/red puncta. These observations suggested that ATRA either promotes autophagosome/lysosome fusion or induces autophagosome acidification (e.g., by the generation of amphisomes). It is worth mentioning that reliable markers to distinguish between early and maturing (acidified) autophagosomes do not exist. In addition, the mechanism by which autophagosomes become acidified is not fully understood; however, fusion with late acidic endosomes has been suggested.

In our efforts to determine the mechanism of retinoic acid-induced autophagosome maturation, we performed several studies using specific antagonists and agonists of the classic retinoic acid receptors (RARs and RXRs). Our observations strongly suggested that the classic RARs and RXRs receptors do not mediate the effects of retinoids on autophagosome maturation. Recent evidence, though has suggested that other retinoid response pathways that are independent of the nuclear receptors may exist. Although the biological significance of retinoic acid binding to alternative intracellular sites is not understood, it was of interest to us that photoaffinity labeling studies have shown direct binding of ATRA to the cation-independent mannose-6-phosphate/IGFII receptor (CI-MPR) with high affinity. CI-MPR is a ubiquitously and constitutively expressed large glycoprotein (~300 kDa).
that plays a fundamental role in endocytosis and degradation of extracellular ligands (IGF-II, uPAR), and sorting and transporting mannose-6-phosphate bearing glycoproteins (such as hydrolases) from the trans-Golgi network (TGN) to endosomes.

To investigate potential effects of retinoids on the intracellular trafficking of CI-MPR, we prepared mGFP- and mRFP-tagged full-length CIMPR constructs. Under basal conditions, the fluorescent CI-MPR fusion proteins are mostly localized in the perinuclear TGN, some vesicular compartments and the plasma membrane. Treatment with retinoids in parallel experiments induces significant redistribution of CI-MPR fusion proteins to peripheral vesicular structures that are not labeled with CFP-LC3 or LAMP-1-RFP (lysosomal marker) but are positive for mCherry-LC3. These data suggested that retinoic acid induces redistribution of CI-MPR into acidified autophagosomes.

To further understand the role of CI-MPR in autophagosome maturation, we utilized siRNA-mediated silencing of endogenous CI-MPR levels. Knockdown of CI-MPR leads to remarkable accumulation of nonacidified immature autophagosomes and Rab9-labeled late endosomes, but has no effect on the abundance of lysosomes. In addition this effect cannot be reversed by retinoic acid treatment, further demonstrating that the effects of these compounds on autophagosome maturation are mediated through CI-MPR. Accumulation of early nonacidified autophagosomes in the absence of CI-MPR indicates the importance of this glycoprotein in autophagosome maturation, but also suggests that autophagosome acidification might be required before fusion with lysosomes. It can be speculated that CI-MPR is
required for acidification of a subset of late endosomes, which in turn mediate autophagosome maturation through fusion. Alternatively, direct translocation of CI-MPR to autophagosomes may be mediating their acidification (Fig.26).

**Fig.26 Proposed Mechanism of autophagosome (AP) maturation by retinoids through redistribution of the cation-independent mannose-6 phosphate receptor (CI-MPR).** Initial formation of APs takes place by engulfing of cargo within the phagophore (PG). The PG elongates and closes to form a vesicle through a process mediated by the LC3 conjugation system. APs then undergo maturation before fusion with lysosomes and formation of autolysosomes. This maturation process potentially relies on acquisition of the vacuolar-type proton ATPase pump that mediates
acidification. This acidification can be accomplished by either fusion of APs with a subset of late endosomes and multivesicular bodies enriched in proton pumps to form amphisomes, or by direct translocation of the proton pump from the TGN to APs. Translocation of the proton pump and other hydrolytic enzymes relies on CI-MPR. Binding of retinoic acid to CI-MPR-enzyme complexes in the TGN induces their translocation to late endosomes (A) or APs (B) and promotes acidification.

Pharmacological modulation of disrupted autophagic activity has been suggested as a strategy for therapies within a wide spectrum of pathological situations including cancer, neurological diseases, premature aging and infectious diseases. Although retinoids are a multitargeting class of compounds that can modulate several physiological and cellular processes, we identified a novel mechanism involving the CI-MPR by which retinoids affect autophagy. This finding can lay the foundation for the development of new and specific retinoid analogues that could enhance or reduce autophagic activity.

**Future Prospective**

We provide here first line of evidences showing that ATRA interacts autophagy pathway by inducing autophagosome maturation. However, there are still many remaining questions and areas that require further characterization. An important remaining aspect that requires further investigation is the identification of the ATRA binding domain on CI-MPR. The extracellular domain of CI-MPR is composed of 15 homologous repeats, (independent structural units of 134-167 aa), displaying multifunctional binding properties. It can be carried out by generating multiple fluorescently-tagged CI-MPR mini-receptors, each lacking one or part of the
repeats, and assess ATRA binding by photo-affinity labeling and co-immunoprecipitation, and ATRA-mediated intracellular redistribution. After generation of these targeted CI-MPR deletion constructs will be expressed in CI-MPR negative cells. These experiments may shed light on to the retinoic binding sites on the CI-MPR and elucidate the mechanism of intracellular redistribution of the CI-MPR by ATRA. These studies will provide crucial insights about the mechanism by which translocation of CI-MPR to AUTs induces maturation.

As our results indicated that retinoids induces AUT acidification in a CI-MPR depended manner. Of great interest, would also be to analyze the consequences of ATRA induced AUT maturation on autophagic activity. To determine if this maturation event actually enhances autophagic activity, we will perform autophagic flux assays and correlate findings with morphological evaluation of the various autophagic compartments by EM. To determine if the ATRA-induced AUT maturation increases their fusion with lysosomes, we will perform in vitro fusion assays, and monitor AUT-LYS fusion with live cell imaging. Follow up studies should aim to characterize the unique proteome of AUTs in ATRA-treated cells under various experimental conditions, examine the interaction of individually identified proteins with CI-MPR and evaluate their effect on autophagy using established methods to measure AUTs, maturation status, and overall autophagic activity.

Also, of particular interest would be to investigate whether autophagosome maturation by ATRA observed in the cultured cells also occurs in vivo in a whole animal. Using morphological and biochemical procedures similar to the ones carried
out throughout the course of this work, we intend to analyze in the transgenic animals the autophagic capabilities of different tissue. To determine the effect of retinoids on autophagy *in vivo* we will subject hemizygous GFP-LC3 transgenic (Tg) mice to diets that will induce VitA deficiency or non-toxic vitamin A excess at various stages of development. The mouse model will be particularly useful to validate the existence of ATRA affects on autophagy in-vivo. Transgenic mice expressing GFP-LC3 (CAG-GFP-LC3) have been used to detect autophagy systemically. However, GFP-LC3 mice cannot distinguish autophagosome biogenesis and maturation events. Also these mice cannot detect autolysosome because GFP-LC3 loses fluorescence due to lysosomal acidic and degradative conditions.

Regarding studying the acidification and maturation in vivo, we can utilize recently develop mCherry-LC3 mice transgenic mice. To resolve above mentioned problems with GFP-LC3 mice, recently transgenic mice (αMyHC-mCherry-LC3) expressing mCherry fused to LC3 under the control of αmyosin heavy chain promoter has been generated. These mice detect autophagy only in cardiomyocytes (Terada et al., 2010). mCherry is an improved-monomeric red-fluorescence protein and does not lose fluorescence under acidic condition. Thus, αMyHC-mCherry-LC3 mice can detect not only autophagosome before fusion with lysosome but also autophagosome after fusion with lysosome. In conclusion, αMyHC-mCherry-LC3 mice can be used in the future studies to detect both autophagosome and autolysosome. This will be an innovative approach to examine the role of autophagy in tissue specific manner. Furthermore, it will be more interesting to look in to the consequences of tissue specific blockage of autophagy by knocking down of CI-MPR using lentiviral RNAi
system. To further analyze the physiological relevance of autophagy in different tissues, we will be looking into the developing models in which autophagy can be studied in specific tissues.

It would be very interesting to study if ATRA also affect other arms of autophagy like CMA. Interestingly, it was recently shown that retinoid compounds selective for particular RARα downstream pathways lead to the selective activation of CMA in the absence of detectable changes in other autophagic pathways (Anguiano et al., 2013). Furthermore, authors have demonstrated that chemical enhancement of CMA with these compounds renders cells more resistant to oxidative stress and proteotoxicity. These findings highlight the potential therapeutic applicability of these or related compounds in the treatment of chronic diseases that associate with loss of CMA activity. It would be very interesting to study if CI-MPR is also involved in the CMA activity under retinoid stimulation.

Identification of the retinoid-binding domain is required to understand the mechanism of CI-MPR redistribution, and will aid in the design of better retinoid analogues with fewer side effects. AUT maturation is a poorly understood process, primarily due to lack of specific cellular markers. The observation that CI-MPR is critical for AUT acidification will aid in understanding the mechanism involved in AUT maturation, and provide means in identifying new markers. Several studies have suggested that reduced autophagy might be responsible for several age-related neurodegenerative diseases, autoimmune disorders, reduced immune function and cancer. Manipulation of the autophagic activity in vivo by retinoids could have
enormous health implications, since they are essential dietary components and frequently used pharmaceuticals.

In Summary, the knowledge we have acquired through the course of these studies will serve as assets as we attempt to learn more of the physiological importance of the nutrient regulation of the different autophagic pathways and contribution of their perturbation during various diseases. Also, by providing the novel mechanism of action for ATRA, it sheds light on potential mechanism of some of the existing retinoids and can be utilized for development of better therapeutics.
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